IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Gwong-Jen J. Chang
Application No. 09/701,536
Filed: June 18, 2001
Confirmation No. 5492
For: NUCLEIC ACID VACCINES FOR PREVENTION OF FLAVIVIRUS INFECTION

FILED VIA EFS

Examiner: Jeffrey S. Parkin, Ph.D.
Art Unit: 1648
Attorney Reference No. 6395-64907-01

FILED VIA ELECTRONIC FILING SYSTEM
UNITED STATES PATENT AND TRADEMARK OFFICE

DECLARATION UNDER 37 C.F.R. § 1.132

1. I, Gwong-Jen J. Chang, Ph.D., am the sole inventor named in the above-referenced patent application. I am the Team Leader of the Molecular Epidemiology & Immunochemistry Lab, Arboviral Diseases Branch, Division of Vector-Borne Infectious Diseases, at the Centers for Disease Control and Prevention (CDC). I have been employed by the CDC for 19 years and have conducted research in the fields of virology and epidemiology for 26 years. I have published more than 58 peer-reviewed scientific articles in the fields of virology, epidemiology and vaccine development. A copy of my current biographical sketch is submitted herewith.

2. I have read and understood the above-referenced patent application, including the pending claims, and the Office action dated March 22, 2007.

3. It is my understanding that in the March 22, 2007 Office action, claims 35-37, 39-45, 47-49, 51-54 and 69 were rejected as allegedly obvious over Phillpotts *et al.* (*Arch. Virol. 141*:743-749, 1996) in view of Kozak (*J. Mol. Biol. 196*:947-950, 1987); and claims 38, 46 and 50 were rejected as allegedly obvious over Phillpotts *et al.* in view of Kozak and Konishi *et al.* (*Virology 188*:714-720,

1992). In addition, it is my understanding that the rejections were based on the conclusion by the Office that it would have been obvious to one of ordinary skill in the art to include a Kozak consensus sequence in the transcriptional unit described by Phillpotts *et al.* to promote optimum expression of the flavivirus antigen.

4. The claimed transcriptional units comprising a prM signal sequence and a Kozak consensus sequence comprising GCCGCCGCC exhibit unexpectedly superior results over the cited references, which is evidenced by the data described in the specification and discussed below.

5. Phillpotts *et al.* describe a St. Louis encephalitis virus (SLEV) construct comprising prM/E (including the prM signal sequence), the CMV immediate early promoter and polyA terminator (pSLE1). The construct does not comprise a Kozak consensus sequence. When 3-4 week old mice were injected intramuscularly (i.m.) with 50 μg of pSLE1, approximately 75% of the mice survived subsequent challenge by SLEV. A second group of mice received the initial 50 μg dose of pSLE1, followed by a booster immunization of 100 μg 21 days later (see page 745 and Figure 1). Approximately 55% of these mice survived challenge by SLEV. In addition, the group receiving the single dose of pSLE1 was evaluated for the presence of neutralizing antibody; however, no neutralizing activity was detected (see page 747). Therefore, vaccination of mice with pSLE1 does not result in complete protection against virus challenge and does not result in the production of neutralizing antibodies.

6. In contrast to the findings of Phillpotts *et al.*, DNA vaccine constructs described in the specification, including pCDJE2-7, pCBJE1-14 and pCIBJES14, exhibit 100% protection against lethal flavivirus challenge after a single vaccinating dose, significant production of neutralizing antibody, and passive protection by maternal antibody. Each of these three exemplary constructs comprises a Kozak consensus sequence comprising GCCGCCGCC, a prM signal sequence, prM/E, the CMV immediate early promoter and a polyA terminator. Example 5 of the specification describes the finding that a single dose of pCDJE2-7 (100 μg) resulted in 100% seroconversion, and JEV-specific antibodies were detectable in vaccinated mice up to 60 weeks following immunization. In addition,

Example 6 describes vaccination of 3-day and 3-week old mice with pCDJE2-7, pCBJE1-14 or pCIBJES14. Mice were administered 50 or 100 μg of the vaccine i.m., and antibody titers were determined three and seven weeks after immunization. All three vaccine constructs elicited seroconversion of 60 (1:1,600 serum dilution) to 100% (1:400 serum dilution) of 3-day and 3-week old mice (see Table 4 in the specification). The data shown in Example 7 demonstrates that vaccination with any of the three constructs results in 100% protection against JEV challenge (see Table 5 of the specification). In addition, pups nursed by female mice vaccinated with pCDJE2-7 were partially to completely protected from JEV challenge by maternal antibody. These results are further described in the Chang *et al.* publication provided as Exhibit A (*J. Virol. 74(9):*4244-4252, 2000). Thus, a single dose of a flavivirus DNA vaccine comprising the claimed transcriptional units provides 100% protective immunity from lethal flavivirus challenge, elicits significant neutralizing antibody titer, and provides passive protection by maternal antibody.

7. The claimed transcriptional units are also superior to other previously described flavivirus DNA vaccine constructs. For example, Lin *et al.* (*J. Virol. 72(1)*:191-200, 1998) describe immunization with a JEV plasmid construct comprising JEV prM/E, the CMV immediate early promoter and a polyA terminator. The construct, referred to as pJME, does not comprise a Kozak consensus sequence. When 3-4 week old mice were administered 3 doses (80 μg/dose at two week intervals) of pJME, only 70% of vaccinated mice survived JEV challenge (see page 195). In addition, JEV-specific antibodies were undetectable by immunoprecipitation and only low antibody titers were detected using a plaque reduction neutralization test (see page 196). Therefore, even with multiple doses, pJME only provides 70% protection against lethal challenge and results in production of little to no neutralizing antibody.

8. Similarly, Konishi *et al.* (*J. Virol. 72(6)*:4925-4930, 1998) describe a JEV DNA vaccine plasmid (called pcDNA3JEME) comprising JEV prM/E, the CMV immediate early promoter and a polyA terminator. pcDNA3JEME further comprises a Kozak sequence; however, the sequence (CGAATTCACC) is different from the Kozak sequence present in the claimed transcriptional units. Konishi *et al.* vaccinated four-week old mice with a single dose (0.1, 1, 10 or 100 μg) of

pcDNA3JEME. At the highest dose (100 μg), 90% of the mice survived JEV challenge, and the mice exhibited low levels of neutralizing antibody. However, none of the mice vaccinated with 0.1, 1, or 10 μg of pcDNA3JEME survived viral challenge, and no neutralizing antibody was detected (see pages 4926-4927). Therefore, even at the highest dose, pcDNA3JEME immunization results in only 90% protection against JEV challenge and low levels of neutralizing antibody.

9. Experiments performed in my laboratory have demonstrated that the efficacy of flavivirus DNA vaccine constructs is not significantly influenced by the presence of a eukaryotic origin of replication or intron sequence; however, a strong promoter (such as the CMV immediate early promoter) and a polyA terminator (such as the BGH polyA) appear to be important (see Example 2 of the specification). In addition, two of the primary differences between the claimed transcriptional units and those described in the prior art are the sequences surrounding the translation initiation site and the composition of the signal peptide preceding the prM protein. I believe these differences significantly contribute to the quantity and quality of the flavivirus antigen synthesized, and therefore, the efficacy of the vaccine. These parameters are discussed in detail in my review article "Flavivirus DNA Vaccines: Current Status and Potential" (Chang *et al. Ann. N.Y. Acad. Sci. 951*:272-85, 2001), provided as Exhibit B. In order to develop a DNA vaccine construct that would result in production of high quantity and quality (*e.g.*, immunogenicity) of flavivirus antigen, I selected the prM signal sequence in combination with a Kozak consensus ribosomal binding sequence comprising GCCGCCGCC. Despite the teachings in the art of other Kozak consensus sequences (such as the sequence utilized by Konishi *et al.*), I selected a sequence comprising GCCGCCGCC. As a result, the flavivirus DNA vaccine constructs comprising the claimed transcriptional units have exhibited unexpectedly superior results over previously described flavivirus vaccine constructs. The superior results are exemplified by the finding that even a single dose of the vaccine results in 100% protection against flavivirus challenge, even in mice as young as 3 days old.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: July 19, 2007

Gwong-Jen J. Chang, Ph.D.

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Chang, Gwong-Jen J.	**Team Leader, Research Microbiologist**

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR	FIELD OF STUDY
National ChungHsing University; Taiwan	Bachelor	1974	Veterinary Medicine
National Taiwan University; Taiwan	Master	1976	Vet. Microbiology
Colorado State University, Fort Collins, CO	Ph.D.	1986	Microbiology/Biochemistry

A. Professional Experience:

1986-1988 **Post-Doctoral Research Associate**, National Research Council, Molecular Biology Branch, Division of Vector-Borne Infectious Diseases (DVBID), CDC, Fort Collins, CO
1988- **Research Microbiologist**, Arbovirus Disease Branch, DVBID, CDC, Fort Collins, CO
1990- **Affiliate Faculty,** Department of Microbiology, Colorado State University, Fort Collins, Colorado
1995- **Term Leader,** Molecular Epidemiology and Immunochemistry Laboratory, Arbovirus Diseases Branch, DVBID, CDC, Fort Collins, CO
2000- **Member of NCID peer-review board**
2007- **Member of DVBID animal use committee**
2004-2006 **NIH study group on Vaccine research and emerging diseases research.**

B. Selected peer-reviewed publications and book chapters.

1. Szu-Chia Hsieh, S.-C., I.-J. Liu, C.-C. King, **G. J. Chang**, and W.-K. Wang. A strong endoplasmic reticulum retention signal in the stem-anchor region of envelope glycoprotein of dengue virus type 2 affects the production of subviral particles. **(Manuscript in preparation).**

2. Chiou, S.-S., W. D. Crill, and **G. J. Chang**. Differential Diagnosis of Japanese Encephalitis Virus Infection Using Cross-Reactive Reduced Virus-Like Particles. **(Manuscript in preparation).**

3. Roberson, J., W. D. Crill and **G. J. Chang**. Differentiation Of West Nile Virus And St. Louis Encephalitis Virus Infections in Serodiagnostic Assays Using Reduced Cross-Reactive Noninfectious Virus-Like Particles. **(Manuscript submitted).**

4. Martin J. E., C. P Theodore, S. Hubka, S. Rucker, I. J. Gordon, M. E. Enama, C. A. Andrews, Q. Xu, B. S. Davis, M. Nason, M. Fay, R. A. Koup, R. T. Bailer, P. L. Gomez, J. R. Mascola, **J. G. Chang**, G. J. Nabel, B. S. Graham and the VRC 302 Study Team. A West Nile Virus DNA Vaccine is Safe and Induces Neutralizing Antibody in Healthy Adults in a Phase I Clinical Trial. J. Infectious Diseases. (in press)

5. Russell B. J., J. O. Velez, J. J. Laven, A. J. Johnson, **G. J. Chang,** R. S. Lanciotti, B. W. Johnson (2007). A Comparison of concentration methods applied to non-infectious flavivirus recombinant antigens for use in diagnostic serological assays. J. Virological Methods. (In press).

6. Daniele B. A. Medeiros, Márcio R. T. Nunes, Pedro F. C. Vasconcelos, **G. J. Chang**, Goro Kuno. (2007). Complete genome characterization of Rocio virus (*Flavivirus*: *Flaviviridae*): A Brazilian flavivirus isolated from a fatal case of encephalitis during an epidemic in São Paulo state. J. General Virol. (In press).

7. Bunning,M L., P. E. Fox, R. A. Bowen, N. Komar, **G. J. Chang**, T. J. Speaker, M. R. Stephens, N. Nemeth, N. A. Panella, S. A. Langevin, P. Gordy, M. Teehee, P. R. Bright, and M. J. Turell. (2007). DNA vaccination of the American Crow (Corvus Brachyrhynchos) provides partial protection against lethal challenge with West Nile virus. Avian Diseases. 51:573–577.

8. Crill, W. D., Trainor, N. and **G. J. Chang**. (2007). A Detailed Mutagenesis Study of Flavivirus Cross-Reactive Epitopes using West Nile Virus-like Particles. J. General Virol. 88:1169-1174.

9. Trainor, N. Crill, W. D., Roberson, J. and **G. J. Chang**. (2007). Mutation Analysis of the Fusion Domain Region of St. Louis Encephalitis Virus Envelope Protein. Virology. 360: 398-406.

10. Chao, D-Y. B. S. Davis and **G. J. Chang**. (2007). Development of Multiplex Real-Time Reverse Transcriptase PCR Assays for Detecting Eight Medically Important Flaviviruses in Mosquitoes. J. Clini. Microbiol. 45:584-589.

11. **Chang, G. J.**, B. S. Davis, C. Stringfield, and C. Lutz. (2007). Prospective immunization of the endangered California condors (Gymnogyps californianus) protects this species from lethal West Nile virus infection. Vaccine. 25:2325-2330.

12. Kuno, G., and **G. J. Chang**. (2007). Full-Length Sequencing and Genomic Characterization of Bagaza, Kedougou, and Zika Viruses. Archive Virol. 158:687-696.

13. Kuno, G., and **G. J. Chang**. (2006). Characterization of Sepik and Entebbe Bat Viruses Closely Related to Yellow Fever Virus. Am. J. Trop. Med. Hyg. 75: 1165-1170.

14. Chien, L.-J. T.-L. Liao, P.-Y. Shu, J.-H. Huang, D. J. Gubler, and **G. J. Chang**. (2006). Development of Real-Time Reverse Transcriptase-PCR Assays to Detect and Serotype Dengue Viruses. J. Clinic. Microbiol. 44:1295-1304.

15. Kuno, G., and **G. J Chang**. (2005) The Biological Transmission of Arboviruses: Re-examination of and New Insights into the Components, Mechanisms, and Unique Traits as well as their Evolutionary Trends. Clinic Microbiol Rev. 18: 606-637.

16. Chao, D.Y., C. C. King, W. K. Wang, W. J. Chen, H. L. Wu, and **G. J Chang**. (2005) Strategically Examining the Full-Genome of Dengue Virus Type 3 in Clinical Isolates Reveals Its Mutation Spectra. Virol. J. 2:71

17. Holmes, D. A., D. E. Purdy, D-Y. Chao, A. J. Noga, and **G. J. Chang**. (2005) Comparative Analysis of the IgM-capture Enzyme-linked Immunosorbent Assay Using Virus-like Particles or Virus-infected Mouse Brain Antigens to Detect IgM Antibody in Patient Serum with Evidence of Flaviviral Infection. J. Clinc. Microbiol. 47:3227-3236.

18. Purdy, D. E. and **G. J. Chang**. (2005) Secretion of Noninfectious Dengue Virus-like Particles and Identification of Amino Acids in the Stem Region Involved in Intracellular Retention of Envelope Protein. Virology. 333:239-250.

19. Nisbert, D. J., K. J. Lee, A. F. van den Hurk, C. A. Johansen, G. Kuno, **G. J. Chang**, J. S. Mackenzie, S. A. Ritchie, and R. A. Hall (2004) Identification of new flaviviruses in the Kokobera virus complex. J. Gen. Virol. 86: 121-124.

20. Crill, W. and **G. J. Chang.** (2004) Localization and characterization of flavivirus envelope glycoprotein cross-reactive epitopes. J. of Virol. 78:13975-13986.

21. Purdy, D. E.; A. J. Noga, and **G. J. Chang**. (2004) A Noninfectious Recombinant Antigen for ELISA Detection of Serum Antibodies to St. Louis Encephalitis Virus. J. Clinic. Microbiol. 42: 4709-4717.

22. **Chang, G. J.**, G Kuno, D. E. Purdy, B. S. Davis. (2004). Recent advancement in flavivirus vaccine development. Expert Rev Vaccines. 3:199-220.

23. Roehrig, J. T., K. E. Volpe, J. Squires, A. R. Hunt, B. S. Davis, and **G. J. Chang**. (2004) Contribution of disulfide bridging to epitope expression of the dengue type 2 virus envelope glycoprotein. J Virol. 78:2648-2652.

24. Austgen, L. E, R. A. Bowen, M. L. Bunning, B. S. Davis, C. J. Mitchell, and **G. J. Chang**. (2004) Experimental infection of cats and dogs with West Nile virus. Emerg Infect Dis. 10:82-86.

25. Turell, M. J., M. Bunning, G. V. Ludwig, B. Ortman, **G. J. Chang**, T. Speaker, A. Spielman, R. McLean, N. Komar, R. Gates,T. McNamara, T. Creekmore, L. Farley, and C. J. Mitchell. (2003) DNA Vaccine for West Nile Virus Infection in Fish Crows (*Corvus ossifragus*). Emerg Infect Dis. 9:1077-1081.

26. **Chang, G. J.**, A. R. Hunt, D. A. Holmes, T. Springfield, T.-S. Chiueh, J. T. Roehrig, D. J. Gubler. (2003) Enhancing biosynthesis and secretion of premembrane and envelope proteins by the chimeric plasmid of dengue virus type 2 and Japanese encephalitis virus. Virology. 306:170-180.

27. **Chang, G. J.**, B. S. Davis, A. R. Hunt,, D. A. Holmes, G. Kuno. (2001) Flavivirus DNA vaccines: current status and potential. Ann. N. Y. Acad. Sci. 951: 272-285.

28. Kuno, G., H. Artsob, N. Karabatsos, K. R. Tsuchiya, and **G. J. Chang**. (2001) Genomic sequencing of deer tick virus and phylogeny of powassan-related viruses of North America. Am J Trop Med Hyg. 65:671-676.

29. Hunt, A. R., C. B. Cropp, and **G. J. Chang**. (2001) A recombinant particulate antigen of Japanese encephalitis virus produced in stably-transformed cells is an effective noninfectious antigen and subunit immunogen. J Virol Methods. 97:133-149.

30. Martin, M., T. F. Tsai, B. Cropp, **G. J. Chang**, D. A. Holmes, J. Tseng, W. Shieh, S. R. Zaki, I. Al-Sanouri, A. F. Cutrona, G. Ray, L. H. Weld, and M. S. Cetron. (2001) Fever and multisystem organ failure associated with 17D-204 yellow fever vaccination: a report of four cases. Lancet. 358:98-104.

31. Davis, B. S., **G. J. Chang**, B. Cropp, J. T. Roehrig, D. A. Martin, C. J. Mitchell, R. Bowen, and M. L. Bunning. (2001) West Nile virus recombinant DNA vaccine protects mouse and horse from virus challenge and expresses in vitro a noninfectious recombinant antigen that can be used in enzyme-linked immunosorbent assays. J Virol. 75:4040-4047.

32. Shieh, W. J., S. M. Jung, C. Hsueh, T. T. Kuo, A. Mounts, U. Parashar, C. F. Yang, J. Guarner, T. G. Ksiazek, J. Dawson, C. Goldsmith, **G. J. Chang**, S. M. Oberste, M. A. Pallansch, L. J. Anderson, and S. R. Zaki. (2001) Pathologic studies of fatal cases in outbreak of hand, foot, and mouth disease, Taiwan. Emerg Infect Dis. 7:146-8.

33. Kuno, G., **G. J. Chang**, K. R. Tsuchiya, and B. R. Miller. (2001) Phylogeny of Thogoto virus. Virus Genes. 23:211-214.

34. Llewellyn, Z. N., X. Ou, **G. J. Chang**, B. Schmitt, M. D. Salman, and R. K. Akkina. (2000) Genetic analysis of vesicular stomatitis virus-New Jersey from the 1995 outbreak in the western United States. Am J Vet Res. 61:1358-1363.

35. **Chang, G. J.**, A. R. Hunt, and B. Davis. (2000) A single intramuscular injection of recombinant plasmid DNA induces protective immunity and prevents Japanese encephalitis in mice. J Virol. 74:4244-4252.

36. Huang, C. Y., S. Butrapet, D. J. Pierro, **G. J. Chang**, A. R. Hunt, N. Bhamarapravati, D. J. Gubler, and R. M. Kinney. (2000) Chimeric dengue type 2 (vaccine strain PDK-53)/dengue type 1 virus as a potential candidate dengue type 1 virus vaccine. J Virol. 74:3020-3028.

37. Tsai, T. F., **G. J. Chang**, and Y. X. Yu. (1999) Japanese encephalitis vaccines, p672-710. *In* A. P. Stanley, and W. A. Orenstein (ed.), Vaccines, 3rd ed. W.B. Saunders, Philadelphia, London, Toronto, Monstreal, Sydney, and Tokyo.

38. Kinney, R. M., M. Pfeffer, K. R. Tsuchiya, **G. J. Chang**, and J. T. Roehrig. (1998) Nucleotide sequences of the 26S mRNAs of the viruses defining the Venezuelan equine encephalitis antigenic complex. Am J Trop Med Hyg. 59:952-964.

39. Kanesa-thasan, N., **G. J. Chang**, B. L. Smoak, A. Magill, M. J. Burrous, and C. H. Hoke Jr. (1998) Molecular and epidemiologic analysis of dengue virus isolates from Somalia. Emerg Infect Dis. 4:299-303.

40. Kuno, G., **G. J. Chang**, K. R. Tsuchiya, N. Karabatsos, and C. B. Cropp. (1998) Phylogeny of the genus Flavivirus. J Virol. 72:73-83.

41. **Chang, G. J.** (1997) Molecular Biology of Dengue Viruses. p175-198. In Dengue/Dengue Hemorrhagic Fever (ed. D. J. Gubler and G. Kuno). CAB International.

42. McFarland, J. M., L. M. Baddour, J. E. Nelson, S. K. Elkins, R. B. Craven, B. C. Cropp, **G. J. Chang**, A. D. Grindstaff, A. S. Craig, and R. J. Smith. (1997) Imported yellow fever in a United States citizen. Clin Infect Dis. 25:1143-1147.

43. Kinney, R. M., S. Butrapet, **G. J. Chang**, K. R. Tsuchiya, J. T. Roehrig, N. Bhamarapravati, and D. J. Gubler. (1997) Construction of infectious cDNA clones for dengue 2 virus: strain 16681 and its attenuated vaccine derivative, strain PDK-53. Virology. 230:300-308.

44. Sullivan, D. G., **G. J. Chang**, and R. K. Akkina. (1997) Genetic characterization of ruminant pestiviruses: sequence analysis of viral genotypes isolated from sheep. Virus Res. 47:19-29.

45. Lin, C. Y., C. C. Lin, **G. J. Chang**, and C. C. King. (1997) Defect of cell-mediated immune response against hepatitis B virus: an indication for pathogenesis of hepatitis-B-virus-associated membranous nephropathy. Nephron. 76:176-185.

46. Kuno, G., C. J. Mitchell, **G. J. Chang**, and G. C. Smith. (1996) Detecting bunyaviruses of the Bunyamwera and California serogroups by a PCR technique. J Clin Microbiol. 34:1184-1188.

47. **Chang, G. J.**, B. C. Cropp, R. M. Kinney, D. W. Trent, and D. J. Gubler. (1995) Nucleotide sequence variation of the envelope protein gene identifies two distinct genotypes of yellow fever virus. J Virol. 69:5773-5780.

48. Ni, H., **G. J. Chang**, H. Xie, D. W. Trent, and A. D. Barrett. (1995) Molecular basis of attenuation of neurovirulence of wild-type Japanese encephalitis virus strain SA14. J Gen Virol. 76:409-413.

49. Brown T. M., **G. J. Chang**, C. B. Cropp, K. E. Robbins, and T. F. Tsai. (1994) Detection of Yellow Fever Virus by Polymerase Chain Reaction. Clin and Diag Virol. 2:41-51.

50. Ku, C. C., C. C. King, C. Y. Lin, H. C. Hsu, L. Y. Chen, Y. Y. Yueh, and **G. J. Chang**. (1994) Homologous and heterologous neutralization antibody responses after immunization with Japanese encephalitis vaccine among Taiwan children. J Med Virol. 44:122-131.

51. Sullivan, D. G., **G. J. Chang**, D. W. Trent, and R. K. Akkina. (1994) Nucleotide sequence analysis of the structural gene coding region of the pestivirus border disease virus. Virus Res. 33:219-228.

52. Weaver, S. C., A. Hagenbaugh, L. A. Bellew, S. V. Netesov, V. E. Volchkov, **G. J. Chang**, D. K. Clarke, L. Gousset, T. W. Scott, D. W. Trent, and a. I. et. (1994) A comparison of the nucleotide sequences of eastern and western equine encephalomyelitis viruses with those of other alphaviruses and related RNA viruses. Virology. 202:1083.

53. Ni, H., N. J. Burns, **G. J. Chang**, M. J. Zhang, M. R. Wills, D. W. Trent, P. G. Sanders, and A. D. Barrett. (1994) Comparison of nucleotide and deduced amino acid sequence of the 5' non-coding region and structural protein genes of the wild-type Japanese encephalitis virus strain SA14 and its attenuated vaccine derivatives. J Gen Virol. 75:1505-1510.

54. **Chang, G. J.**, D. W. Trent, A. V. Vorndam, E. Vergne, R. M. Kinney, and C. J. Mitchell. (1994) An integrated target sequence and signal amplification assay, reverse transcriptase-PCR-enzyme-linked immunosorbent assay, to detect and characterize flaviviruses. J Clin Microbiol. 32:477-483.

55. Lewis, J. A., **G. J. Chang**, R. S. Lanciotti, R. M. Kinney, L. W. Mayer, and D. W. Trent. (1993) Phylogenetic relationships of dengue-2 viruses. Virology. 197:216-224.

56. Kinney, R. M., **G. J. Chang**, K. R. Tsuchiya, J. M. Sneider, J. T. Roehrig, T. M. Woodward, and D. W. Trent. (1993) Attenuation of Venezuelan equine encephalitis virus strain TC-83 is encoded by the 5'-noncoding region and the E2 envelope glycoprotein. J Virol. 67:1269-1277.

57. Lewis, J. G., **G. J. Chang**, R. S. Lanciotti, and D. W. Trent. (1992) Direct sequencing of large flavivirus PCR products for analysis of genome variation and molecular epidemiological investigations. J Virol Methods. 38:11-23.

58. Lanciotti, R. S., C. H. Calisher, D. J. Gubler, **G. J. Chang**, and A. V. Vorndam. (1992) Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol. 30:545-551.

59. Nitayaphan, S., J. A. Grant, **G. J. Chang**, and D. W. Trent. (1990) Nucleotide sequence of the virulent SA-14 strain of Japanese encephalitis virus and its attenuated vaccine derivative, SA-14-14-2. Virology. 177:541-552.

60. Roehrig, J. T., A. R. Hunt, **G. J. Chang**, B. Sheik, R. A. Bolin, T. F. Tsai, and D. W. Trent. (1990) Identification of monoclonal antibodies capable of differentiating antigenic varieties of eastern equine encephalitis viruses. Am J Trop Med Hyg. 42:394-398.

61. **Chang, G. J.**, B. J. Johnson, and D. W. Trent. (1988) Site-specific oligonucleotide-directed mutagenesis using T4 DNA polymerase. DNA. 7:211-217.

62. **Chang, G. J.**, and D. W. Trent. (1987) Nucleotide sequence of the genome region encoding the 26S mRNA of eastern equine encephalomyelitis virus and the deduced amino acid sequence of the viral structural proteins. J Gen Virol. 68:2129-2142.

C. Honor:

2001	Secretary's Award for Distinguish Service
2002	Center for disease Control and Prevention, Charles C. Shepard Science Award for Laboratory and Methods
2002	National Center for Infectious Diseases, James H. Nakano Citation
2003	South East Federal laboratory Consortium, 2003 Excellence in Technology Transfer Award
2004	National Federal laboratory Consortium, 2004 Excellent in Technology Transfer Award

D. Patents and Pending Patents.

Nucleic Acid Vaccines for Prevention of Flavivirus Infections:

Australian patent number: 778988

New Zealand patent number: 1509

South Africa patent number: 2003/7580

U.S. patent number: 7,227,011

Chinese patent number: ZL02807758.X

EU. Patent number: 1084252

Pending Patents.

Nucleic Acid Vaccines for Prevention of Flavivirus Infections:

11 additional Countries and EU

Localization and Characterization of Flavivirus Envelope Glycoprotein Cross-reactive Epitopes and Methods for Their Use

US PCT filled on July/27/2005

JOURNAL OF VIROLOGY, May 2000, p. 4244–4252
0022-538X/00/$04.00+0

Vol. 74, No. 9

A Single Intramuscular Injection of Recombinant Plasmid DNA Induces Protective Immunity and Prevents Japanese Encephalitis in Mice

GWONG-JEN J. CHANG,* ANN R. HUNT, AND BRENT DAVIS

Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service, U.S. Department of Health and Human Services, Fort Collins, Colorado 80522

Received 10 November 1999/Accepted 1 February 2000

Plasmid vectors containing Japanese encephalitis virus (JEV) premembrane (prM) and envelope (E) genes were constructed that expressed prM and E proteins under the control of a cytomegalovirus immediate-early gene promoter. COS-1 cells transformed with this plasmid vector (JE-4B clone) secreted JEV-specific extracellular particles (EPs) into the culture media. Groups of outbred ICR mice were given one or two doses of recombinant plasmid DNA or two doses of the commercial vaccine JEVAX. All mice that received one or two doses of DNA vaccine maintained JEV-specific antibodies 18 months after initial immunization. JEVAX induced 100% seroconversion in 3-week-old mice; however, none of the 3-day-old mice had enzyme-linked immunosorbent assay titers higher than 1:400. Female mice immunized with this DNA vaccine developed plaque reduction neutralization antibody titers of between 1:20 and 1:160 and provided 45 to 100% passive protection to their progeny following intraperitoneal challenge with 5,000 PFU of virulent JEV strain SA14. Seven-week-old adult mice that had received a single dose of JEV DNA vaccine when 3 days of age were completely protected from a 50,000-PFU JEV intraperitoneal challenge. These results demonstrate that a recombinant plasmid DNA which produced JEV EPs in vitro is an effective vaccine.

Japanese encephalitis (JE) is a mosquito-borne viral disease of major public health importance in Asia. More than 35,000 cases and 10,000 deaths are reported annually (52). *Japanese encephalitis virus* (JEV) is a member of the genus *Flavivirus* in the family *Flaviviridae*. More than 70 species in the *Flavivirus* genus have been genetically and serologically classified (29). Other important human pathogenic flaviviruses include yellow fever, dengue type 1 to 4 (DEN1 to DEN4), tick-borne encephalitis (TBE), and St. Louis encephalitis (SLE) viruses. Vaccination has been an effective mechanism for prevention of flavivirus infection in humans and domestic animals. Three JEV vaccines are in widespread production and use (52). These are inactivated virus from infected mouse brain, inactivated virus from primary hamster kidney cells, and a live attenuated SA14-14-2 vaccine. Only inactivated JEV vaccine, JEVAX, produced in mouse brain is distributed commercially and available internationally (52). Inactivated, mouse brain-derived whole virus vaccine is costly to prepare and carries the risk of allergic reaction to murine encephalitogenic basic proteins or gelatin stabilizer (45; M. M. Andersen, and T. Ronne, Letter, Lancet 337:1044, 1991). Since 1989, an unusual number of systemic reactions characterized by generalized urticaria and/or angioedema following JEVAX immunization have been reported from Australia, Canada, and Denmark (36). A major problem associated with use of the inactivated mouse brain vaccine is the failure to stimulate long-term immunity (39). Multiple immunization is recommended to provide adequate protection (28, 39). The attenuated JEV vaccine, SA14-14-2, is undergoing clinical trials (31). However, because of regulatory issues this vaccine has not found wide acceptance outside the People's Republic of China (11).

* Corresponding author. Mailing address: P.O. Box 2087, Division of Vector-Borne Infectious Diseases, CDC, Foothill Campus, Fort Collins, CO 80522-2087. Phone: (970) 221-6497. Fax: (970) 221-6476. E-mail: gxc7@cdc.gov.

Several experimental recombinant virus, attenuated virus, and subunit JEV vaccines have been reported. Recombinant baculovirus vector that contained the JEV envelope (E) protein gene has been used to infect insect cells and produce E protein that has been studied as a biosynthetic immunogen (33). Recombinant vaccinia viruses expressing the JEV genes extending from premembrane (prM) to NS2B proteins have been the most promising candidate vaccines. These candidate vaccines produced extracellular virus-like particles (EPs) in infected cell culture that induced high titers of neutralizing and hemagglutination-inhibiting antibodies and protective immunity in mice (19–21, 47, 54). Recombinant vaccinia viruses expressing the same JEV genes based on the attenuated vaccinia virus strain, NYVAC-JEV, or canarypox, ALVAC-JEV, were tested in phase I human trials (18). In this trial, only 1 in 10 ALVAC-JEV recipients developed detectable viral neutralizing antibody, and vaccinia virus-preimmune recipients had a significantly lower humoral immune response.

Inoculation of animals with purified plasmid vectors (DNA) by the intramuscular (i.m.) or intradermal route leads to expression of the recombinant vector-encoded protein in transfected cells, resulting in stimulation of a protein-specific immune response. Plasmid DNA vaccines provide an alternative to attenuated, inactivated, or virus-vectored subunit vaccines. Flavivirus DNA vaccines for Murray Valley encephalitis, DEN2, JE, SLE, and TBE (Central European encephalitis and Russian spring summer encephalitis) viruses have been developed and tested in the mouse model (4, 17, 24, 30, 38, 49). All of these plasmid DNA constructs contained similar transcriptional regulatory elements and a flavivirus gene cassette. Vaccination of mice with these plasmid DNA vaccines induced a virus-specific antibody response, as detected by enzyme-linked immunosorbent assay (ELISA). However, production of neutralizing antibody leading to 100% protection of vaccinated animals from virus challenge was observed only after multiple immunizations or delivery of DNA to the epidermis by particle

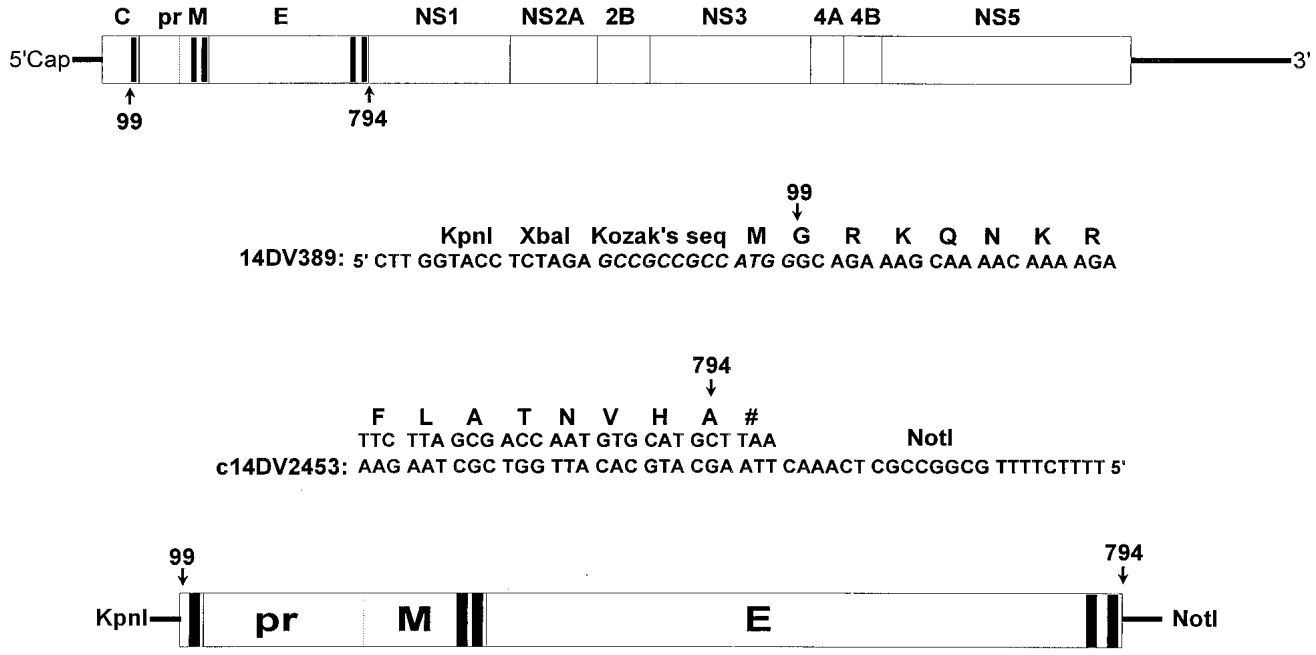


FIG. 1. Map of the JEV genomic structure (top) and the DNA sequence of oligonucleotides used in RT-PCR to construct the transcription unit for the expression of prM-E protein coding regions (bottom). Potential transmembrane helices of viral polyprotein are indicated by blackened areas.

bombardment (4, 24, 49). In this study, we constructed a JEV prM and E gene cassette that incorporates an extended signal peptide sequence at the NH_2 terminus of the prM gene and Kozak's sequence, an optimal translation enhancing element surrounding the AUG site. JEV protein expression was characterized using six different recombinant vectors containing the same insert. The humoral immune response and protection from virulent JEV challenge following immunization with the recombinant plasmid DNAs were compared to findings for the human vaccine, JEVAX, licensed by the U.S. Food and Drug Administration, in outbred ICR mice.

MATERIALS AND METHODS

Cell culture and virus strain. COS-1, COS-7, and SV-T2 cells (1650-CRL, 1651-CRL, and 163.1-CCL; American Type Culture Collection) were grown at 37°C in Dulbecco's modified Eagle medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 7.5% $NaHCO_3$ (30 ml/liter), penicillin (100 U/ml), streptomycin (100 μg/ml). COS-1 and COS-7 cells were derived from simian virus 40 (SV40) transformed CV1 cells which have an African green monkey kidney cell origin. SV-T2 cells were derived from SV40-transformed mouse fibroblasts. Vero cells were grown under the same conditions except that 5% fetal calf serum without nonessential amino acid was used. C6/36 cells (13) were grown at 28°C in the same medium used for the COS-1 cells. The SA14 strain of JEV, propagated by intracranial inoculation into suckling mouse brain, was used for animal challenges and plaque reduction neutralization tests (PRNT). The SA14 virus used in ELISA and Western blot experiments was propagated in C6/36 cells and purified by ultracentrifugation on 30% glycerol–45% potassium tartrate gradients (37).

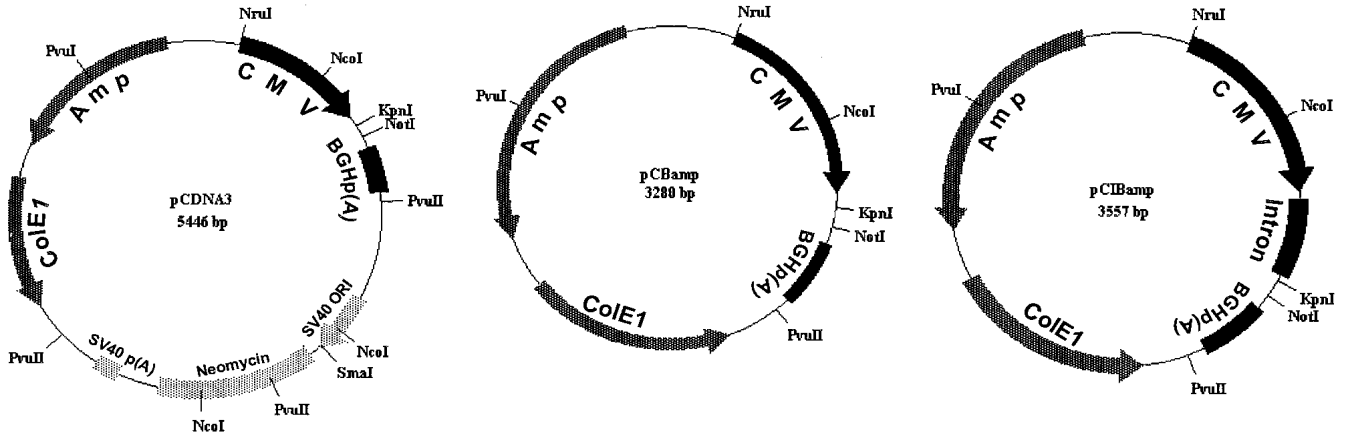
Construction of plasmids expressing JEV prM and E gene proteins. Genomic RNA was extracted from 150 μl of SA14 mouse brain JEV by using a QIAamp viral RNA kit (Qiagen, Santa Clarita, Calif.). RNA was adsorbed on a silica membrane, eluted in 80 μl of diethyl pyrocarbonate (Sigma Chemical Co., St. Louis, Mo.)-treated water, and used as a template for amplification of JEV prM and E genes. Primer sequences were obtained from the published data (35). A single cDNA fragment containing genomic nucleotides (nt) 389 to 2478 was amplified by reverse transcriptase-mediated PCR (RT-PCR). Restriction enzyme sites for *Kpn*I and *Xba*I and Kozak's sequence for an optimal translation initiation (25, 26) were engineered at the 5′ terminus of the cDNA by amplimer 14DV389. An in-frame translation termination codon, followed by a *Not*I restriction site, was introduced at the 3′ terminus of the cDNA by amplimer c14DV2453 (Fig. 1). A single-tube RT-PCR was performed using a Titan RT-PCR Kit (Roche Molecular Biochemical, Indianapolis, Ind.). The RT-PCR product was purified using a QIAquick PCR purification kit (Qiagen), and the DNA was eluted with 50 μl of 1 mM Tris-HCl (pH 7.5).

All vector constructions and analyses were carried out using standard techniques (46). RT-PCR-amplified cDNA was digested with enzymes *Kpn*I and *Not*I and inserted into the *Kpn*I-*Not*I site of eukaryotic expression plasmid vector pCDNA3 (Invitrogen, Carlsbad, Calif.). Electroportion-competent *Escherichia coli* XL1-Blue cells (Stratagene, La Jolla, Calif.) were transformed by electroporation (Gene Pulser; Bio-Rad Laboratories, Hercules, Calif.) and plated on Luria broth (LB) agar plates that contained carbenicillin (100 μg/ml; Sigma). Clones were picked and inoculated into 3 ml of LB containing carbenicillin (100 μg/ml). Plasmid DNA was extracted from a 14-h LB culture by using a QIAprep Spin Miniprep kit (Qiagen). Automated DNA sequencing was performed as recommended on an ABI Prism 377 DNA sequencer (Perkin-Elmer/Applied Biosystems, Foster City, Calif.). Both strands of the cDNA were sequenced and compared to the published SA14 virus sequence (35).

The pCDNA3 fragment from nt 1289 to nt 3455, which contained the f1-encoded eukaryotic origin of replication (ori), SV40 ori, neomycin coding region, and SV40 poly(A) elements, was deleted by *Pvu*II digestion and then self-ligated to generate plasmid pCBamp. The pCIBamp vector, which contained a chimeric intron insertion at the *Nco*I-*Kpn*I site of the pCB vector, was constructed by excising the intron sequence from pCI (Promega, Madison, Wis.) by digestion with *Nco*I and *Kpn*I. The resulting 566-bp fragment was cloned into *Nco*I-*Kpn*I-digested pCBamp to replace its 289-bp fragment. Figure 2 shows a schematic drawing of plasmids pCDNA3, pCBamp, and pCIBamp.

The DNA fragment containing the JEV coding region in the recombinant plasmid pCDJE2-7, derived from the pCDNA3 vector, was excised by *Not*I and *Kpn*I or *Xba*I digestion and cloned into the *Kpn*I-*Not*I sites of pCB, pCIB, pCEP4 (Invitrogen), and pREP4 (Invitrogen) and into the *Spe*I-*Not*I site of the pRc/RSV (Invitrogen) expression vector to create pCBJE1-14, pCIBJES14, pCEJE, pREJE, and pRCJE, respectively. Both strands of the cDNA from each plasmid vector were sequenced, and recombinant clones with a correct nucleotide sequence were identified. Plasmid DNA for in vitro transformation or mouse immunization was purified by anion-exchange chromatography using an Endo-Free Plasmid Maxi kit (Qiagen).

IFA. Expression of JEV-specific gene products by the various recombinant expression plasmids was evaluated by indirect immunofluorescence antibody assay (IFA) in the transient expression system using COS-1, COS-7, and SV-T2 cells. For transformation, cells were grown to 75% confluence in 150-cm^2 culture flasks, trypsinized, and resuspended in 4°C phosphate-buffered saline (PBS) to a final density of 1×10^7 to 2×10^7 cells/ml. Five hundred microliters of cell suspension was then electroporated with 10 μg of plasmid DNA, using a Bio-Rad Gene Pulser II set at 250 V and 960 μF. Cells were diluted with 25 ml of fresh medium after electroporation and seeded into one 75-cm^2 flask. Forty-eight hours after transformation, the medium was removed, and the cells were



TATA box KpnI BamHI EcoRI EcoRV NotI

TATATAA...(92nt)...*GGTACC*GAGCTC*GGATCC*ACTAGTAACGGCCGCCAGTGTGCTG*GAATTC*TGCA*GATATC*CATCACACTG*GCGGCCGC*...BHG-poly(A)

FIG. 2. Schematic representations of plasmid vectors pCDNA3, pCBamp, and pCIBamp. These plasmids include the CMV promoter/enhancer element, BGH poly(A) signal and transcription termination sequence [BHGp(A)], ampicillin resistance gene (Amp), and ColE1 ori for selection and maintenance in *E. coli*. The f1 ori for single-stranded rescue in *E. coli* cells, SV40 ori, neomycin coding region, and SV40 poly(A) [SV40 p(A)] sequences were deleted from pCDNA3 to generate pCBamp. An intron sequence was inserted in the *Nco*I-*Kpn*I site of pCBamp to generate pCIBamp. The multiple cloning site for the insertion of JEV genes, located between the TATA box of the CMV promoter/enhancer and BHG poly(A) site, is shown.

trysinized and resuspended in 5 ml of PBS with 3% normal goat serum. Ten-microliter aliquots of the cell suspension were then spotted onto slides, air dried, and fixed with acetone at 4°C for 10 min. Immunofluorescent mapping of the E protein-specific epitopes was performed using a panel of murine monoclonal antibodies (MAbs) (15, 42, 55) and JEV-specific hyperimmune mouse ascitic fluid (HIAF). All antibodies were tested at 1:400 dilution in PBS.

Selection of an in vitro-transformed stable cell line constitutively expressing JEV-specific gene products. COS-1 cells transformed with 10 μg of pCDJE2-7 DNA by electroporation were incubated in nonselective culture medium for 24 h and then treated with neomycin (G418; 0.5 mg/ml; Sigma). G418-resistant colonies, which became visible after 2 to 3 weeks, were cloned by limited dilution in G418-containing medium. Expression of the JEV proteins was determined by IFA using JEV HIAF. One IFA-positive (JE-4B) and one IFA-negative (JE-5A) clone were selected for further analysis and maintained in medium containing 200 μg of G418 per ml. These stably transformed cells secreted antigen in the form of EPs (A. Hunt and G. J. Chang, unpublished data).

Antigen capture ELISA for detection of E protein secreted into culture fluid. The antigen capture ELISA, a modification of the procedure described by Guirakhoo et al. (8), was used to detect E protein from transiently transformed cells or JE-4B culture fluid. Flavivirus group-reactive MAb 4G2 was used to capture the JEV antigens (7). The 4G2-captured antigen was detected using horseradish peroxidase-conjugated MAb 6B6C-1 by incubation for 1 h at 37°C. Enzyme activity on the solid phase was detected with 3,3′,5,5′-tetramethylbenzidine ELISA substrate (Life Technologies, Grand Island, N.Y.); the reaction was stopped with the addition of 2 M H_2SO_4, and the optical density was measured at 450 nm.

Mouse experiments. Three-day-old mixed-sex or 3-week-old female ICR outbred mice were vaccinated i.m. with 50 or 100 μg of plasmid DNA at a concentration of 1 μg/μl in PBS or subcutaneously (s.c.) with 1/10 or 1/5 of the adult human dose of JEVAX (manufactured by the Research Foundation for Microbial Disease of Osaka University and distributed by Connaught Laboratories, Swiftwater, Pa.). The chloramphenicol acetyltransferase (CAT) protein expression plasmid pCDNA3/CAT (Invitrogen) was used as the vaccination control. Selected groups of mice were boosted 3 weeks later with an additional dose of plasmid vaccine or JEVAX. Mice were bled from the retro-orbital sinus; serum samples were evaluated for JEV antibody by ELISA and Western blotting using purified JEV and by PRNT.

Mice vaccinated at 3 days of age were challenged intraperitoneally (i.p.) 7 weeks postvaccination with JEV strain SA14 (50,000 PFU/100 μl) and observed for 3 weeks. To evaluate passive protection by maternal antibody, pups were obtained from mating of nonimmunized males with immunized females 9 weeks following their vaccination with plasmid DNA at 3 weeks of age. Pups were challenged by the i.p. route 3 to 15 days after birth with SA14 virus (5,000 PFU/100 μl) and observed daily for 3 weeks. Postchallenge serum was collected from survivors and tested for reactivity with JEV antigens by ELISA and Western blotting.

Serological tests. Postvaccination and postchallenge serum samples were tested for the ability to bind to purified JEV by ELISA, neutralize JEV infectivity by PRNT, or recognize JEV proteins by Western blotting (12, 41, 48). The PRNT assay was performed by incubating ~200 PFU of SA14 virus in 100 μl of Dulbecco's modified Eagle medium containing 5% bovine serum albumin and 20 mM HEPES buffer (pH 8.0) with serial twofold dilutions of serum specimens, started at 1:10, in 100 μl of the same buffer in 96-well trays at 4°C overnight. Serum specimens were heat inactivated at 56°C for 30 min before use. Duplicate 100-μl aliquots were assayed for infective virus by plaque formation on Vero cell monolayers. The percent plaque reduction was calculated relative to virus controls without serum. Titers were expressed as the reciprocal of serum dilutions yielding a 90% reduction in plaque number ($PRNT_{90}$).

RESULTS

Effect of the promoter and poly(A) signal on the efficiency of JEV prM and E protein expression. Four eukaryotic cell expression plasmids that contained the JEV coding region extending from genomic nt 390 to nt 2478 were constructed. This region of the genome encoded the prM and E genes. The Kozak sequence for the eukaryotic translation initiation site (underlined) of −9 to +4, GCCGCCGCC**ATG**G, at the 5′ terminus (2, 25, 26, 27) and the in-frame translation terminat ion sequence at the 3′ terminus of cDNA were incorporated directly into cDNA by RT-PCR using viral RNA as a template. Transcription of the JEV genes in plasmid pCDJE2-7 was controlled by the human cytomegalovirus (CMV) early IA gene promoter/enhancer. The resulting mRNA is terminated and stabilized by a bovine growth hormone (BGH) transcript ion terminator and a poly(A) signal, respectively. The transcriptional control elements in pREJE were replaced by the Rous sarcoma virus (RSV) long terminal repeat promoter and SV40 poly(A). The pCEJE and pRCJE plasmids contain CMV plus SV40 poly(A) and RSV plus BGH poly(A), respectively (Table 1).

To determine the influence of the promoter and poly(A) elements on JEV prM and E protein expression, recombinant plasmids pCDJE2-7, pCEJE, pRCJE, and pREJE were ini-

TABLE 1. Transient expression of JEV prM and E proteins by various recombinant plasmids in two transformed cell lines

Name	Promoter	Intron	Poly(A)	Ori	Recombinant plasmid	IFA intensity/% positive[a]	
						COS-1	COS-7
pCDNA3	CMV	No	BGH	SV40	pCDJE2-7	3+/40	3+/35
pCBamp	CMV	No	BGH	No	pCBJE1-14	3+/45	ND
pCIBamp	CMV	Yes	BGH	No	pCIBJES14	3+/39	ND
pCEP4	CMV	No	SV40	OriP	pCEJE	2+/4	2+/3
pREP4	RSV	No	SV40	OriP	pREJE	1+/3	1+/2
pRc/RSV	RSV	No	BGH	SV40	pRCJE	1+/3	1+/3
pCDNA3	CMV	No	BGH	SV40	pCDNA3/CAT	−	−

[a] Various cell lines were transformed with pCDNA3/CAT (negative control), pCDJE2-7, pCBJE1-14, pCIBJES14, pCEJE, pREJE, or pRCJE. Cells were trypsinized 48 h later and tested by IFA with JEV HIAF. Data are presented as the intensity (scale of 1+ to 4+) and percentage of IFA-positive cells. pCDNA3/CAT-transformed cells were used as the negative control. ND, not determined. −, negative.

tially tested for the ability to express JEV prM and E proteins following transformation of various mammalian cells. COS-1, COS-7, and SV-T2 cells were transiently transformed with equal amounts of pCDJE2-7, pCEJE, pRCJE, or pREJE plasmid DNA. The SV-T2 cell line was excluded from further testing after preliminary results showed that less than 1% of pCDJE2-7-transformed SV-T2 cells were expressing JEV antigen.

JEV antigens were expressed in COS-1 and COS-7 cells transformed by all four recombinant plasmids, thus confirming that the CMV or RSV promoter and BGH or SV40 poly(A) elements were functionally active. However, the percentage of transformed cells and the level of JEV antigens expressed, as determined by the number of IFA-positive cells and IFA intensity, respectively, differed significantly (Table 1). A significantly higher percentage of pCDJE2-7-transformed COS-1 cells expressed JEV proteins with greater IFA intensity at a level equal to that observed with JEV-infected cells. Cells transformed with the pCEJE, pREJE, or pRCJE vector, on the other hand, showed a lower percentage of antigen-expressing cells as well as a lower IFA intensity. Vectors containing the CMV promoter and BGH poly(A) were selected for further analysis (Fig. 2).

To determine whether the enhanced expression of JEV proteins by the pCDJE2-7 vector was influenced by the SV40 ori, we constructed the pCBJE1-14 vector in which a 2,166-bp fragment containing the f1 ori, SV40 ori, neomycin coding region, and SV40 poly(A) elements was deleted. A chimeric intron was then inserted into pCBJE1-14 to generate pCIBJES14. Plasmid pCIBJES14 was used to determine whether the expression of JEV proteins could be enhanced by an intron sequence. Following transformation, both pCBJE1-14 and pCIBJES14 vectors resulted in cells expressing levels of JEV proteins similar to that observed with the pCDJE2-7 vector (Table 1). These results indicated that expression of the JEV proteins was influenced only by the transcriptional regulatory elements encoded in the recombinant plasmid. Neither the SV40 ori nor the intron sequence enhanced JEV protein expression in the cells used.

Epitope mapping of E protein expressed by a stably transformed cell line constitutively expressing JEV-specific gene products. Authenticity of the JEV E protein expressed by the JE-4B clone was demonstrated by epitope mapping by IFA using a panel of JEV E-specific murine MAbs. JEV HIAF and one irrelevant mouse ascitic fluid were used as positive and negative antibody controls, respectively. Four JEV-specific, six flavivirus subgroup-specific, and two flavivirus group-reactive MAbs reacted similarly with the 4B clone and with JEV-infected COS-1 cells (Table 2).

Detection of JEV E protein secreted by the JE-4B COS-1 cell line. An antigen capture ELISA, employing flavivirus group-reactive, anti-E MAbs 4G2 and 6B6C-1, was used to detect JEV E proteins that were secreted into the culture fluid by the COS-1 cell clone JE-4B. Antigen could be detected in the culture fluid the first day following seeding of the cells with maximum ELISA titers that ranged from 1:16 to 1:32.

Comparison of immune responses in mice vaccinated with pCDJE2-7 genetic vaccine and JEVAX. Plasmid pCDJE2-7 was used as a nucleic acid vaccine to induce an antibody response in mice by immunizing groups of five 3-week-old female ICR outbred mice. Mice were bled at 3, 6, 9, 23, 40, and 60 weeks after immunization, and antibody titers were determined by ELISA or by PRNT. As expected, sera from animals in the pCDNA3/CAT control group did not contain JEV antibody. All animals immunized with pCDJE2-7 and JEVAX seroconverted by 3 weeks after the first vaccination (Table 3). The antibody titers were similar irrespective of the number of doses

TABLE 2. Epitope mapping of E protein expressed by JE-4B, a pCDJE2-7 stably transformed clone of COS-1 cells, with JEV-reactive antibodies[a]

MAb or antiserum	Biological activity of MAb		IFA intensity of cells	
	Specificity	Biological function	JEV infected	4B
MAbs				
MC3	JEV specific		2+	2+
2F2	JEV specific	HI, N	4+	4+
112	JEV specific		4+	4+
503	JEV specific	N	4+	3+
109	Subgroup	HI	2+	1+
N.04	Subgroup	HI, N	3+	4+
201	Subgroup		1+	1+
203	Subgroup		4+	3+
204	Subgroup		2+	2+
301	Subgroup	HI	2+	2+
504	Flavivirus		4+	4+
6B6C-1	Flavivirus		2+	2+
3B4C-4	VEE		−	−
HIAF				
Anti-JEV			4+	3+
Anti-WEE			−	−
PBS			−	−

[a] VEE, Venezuelan equine encephalomyelitis virus; WEE, Western equine encephalomyelitis virus. −, negative.

TABLE 3. Persistence of the immune response in mice (five per group) immunized with pCDJE2-7 or JEVAX

Inoculation[a]	ELISA titer (log_{10})						$PRNT_{90}$ titer		
	3[b]	6	9	23	40	60[c]	3	6	9
pCDJE2-7									
1 dose	2.6–3.2	3.8–5.0	3.8–4.4	>3.2	>3.2	2.4, 2.4, 3.8, 4.4	<20	20	40–160
2 doses	2.6–3.8	4.4	3.8–4.4	>3.2	>3.2	2.6, 3.8, 3.8	<20	20–40	40–160
JEVAX, 2 doses	2.6–3.8	4.4–5.0	3.8–5.6	>3.2	>3.2	<2, <2, <2, 4.4	<20	20–40	20–160
pCDNA3/CAT, 2 doses	<100	<100	<100	ND[d]	ND	ND	<20	<20	<20

[a] Three-week-old mice were inoculated i.m. with one or two 100-μg doses of plasmid DNA or twice s.c. with one-fifth of the human dose of JEVAX.
[b] Weeks postimmunization.
[c] Individual serum titers.
[d] ND, not determined.

of pCDJE2-7 or JEVAX given. Mouse serum samples collected 9 weeks after immunization were also tested by Western blotting using purified JEV. Serum specimens from DNA-vaccinated mice, which had reactivity similar to that of JEV HIAF, detected E and prM proteins (Fig. 3). However, mouse serum from JEVAX-immunized mice reacted only with E protein. Comparable ELISA antibody titers were maintained in DNA-vaccinated groups for up to 60 weeks, at which time the experiment was terminated. Only one of four mice in the JEVAX group remained JEV antibody positive at 60 weeks postinoculation. These results demonstrated that one dose of JEV-specific nucleic acid vaccine was more effective in maintaining JEV antibody levels in mice than the commercially available vaccine JEVAX.

Comparison of various nucleic acid vaccine constructs and JEVAX for ability to induce JEV-reactive antibody in different age groups of mice. Similar amounts of JEV protein were expressed by COS-1 cells transformed by either pCDJE2-7, pCBJE1-14, or pCIBJES14. JEV antibody induction by these nucleic acid constructs was compared to results for JEVAX in two different age groups of mice. Three-day-old mixed-sex or 3-week-old female ICR outbred mice, 10 per group, were vaccinated i.m. with 50 or 100 μg of plasmid DNA or s.c. with 1/10 or 1/5 of the adult human dose of JEVAX, respectively. Serum specimens were collected at 7 weeks after immunization and tested at 1:400 or 1:1,600 by ELISA. Ninety to 100% of all 3-week-old mice that received pCBJE1-14, pCDJE2-7, pCIBJES14, or JEVAX had antibody titers of ≥1:1,600. However, a significant difference in antibody response was observed in 3-day-old groups that received various vaccines. None of the 3-day-old JEVAX-vaccinated mice had antibody titers higher than 1:400. All 3-day-old mice vaccinated with pCBJE1-14 had antibody titers higher than 1:1,600. Seroconversion of 100% was observed at 1:400 in 3-day-old mice that received pCDJE2-7 or pCIBJES14, but only 60% of both mouse groups were positive at 1:1,600. pCBJE1-14 was the most effective of three DNA constructs tested. The minimum dose of this DNA construct capable of providing 100% seroconversion (1:400 by ELISA) by i.m. immunization in 3-week-old mice was determined to be 25 μg (data not shown).

Protective immunity conferred by the nucleic acid vaccine. Mice immunized at 3 days of age were challenged by the i.p. route at 7 weeks postvaccination with the SA14 strain of JEV (50,000 PFU/100 μl) and observed for 3 weeks. One hundred percent of the animals that received various nucleic acid vaccine constructs were protected. In contrast, only 40 and 30% of mice that received JEVAX and pCDNA3/CAT, respectively, survived virus challenge (Fig. 4). These results suggested that the DNA vaccine could be effective as a neonatal vaccine. In contrast, JEVAX was not as effective in neonatal animals.

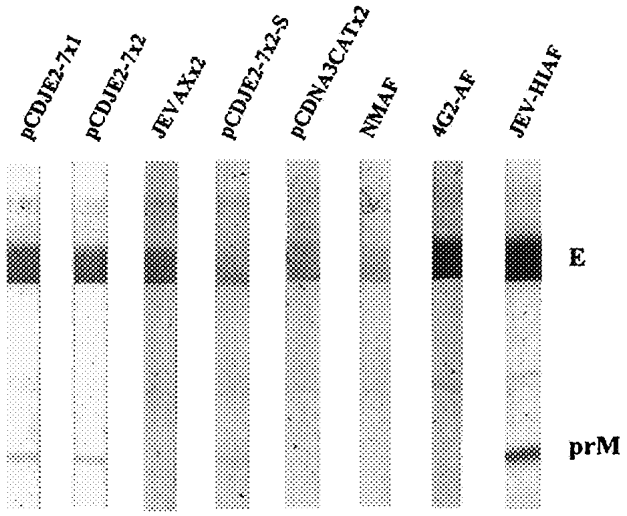


FIG. 3. JEV-specific reactivity of prechallenge and postchallenge serum samples obtained from mice immunized with DNA vaccine or JEVAX. Serum specimens collected from the mice used in the experiments represented in Tables 3 and 4 were randomly selected and tested at 1:1,000 dilution by Western blot analysis using purified JEV as the antigen. pCDJE2-7x2-S was the serum from one of the mice challenged at 4 days of age (Table 4). NMAF, 4G2-AF, and JEV HIAF were the mouse ascitic fluids included as normal mouse, E-specific, and JEV hyperimmune controls, respectively.

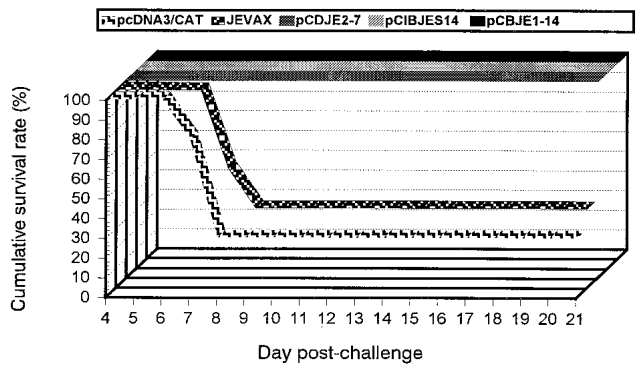


FIG. 4. Postchallenge survival rates of mice (10 per group) that were immunized with pCDJE2-7, pCBJE1-14, pCIBJES14, pcDNA3/CAT, or JEVAX at 3 days of age and challenged i.p. with 50,000 PFU of JEV (SA14) 7 weeks postimmunization. A P value of 0.003 was obtained by Fisher's exact test when the survival rate of the JEV DNA-immunized groups was compared with that of the pcDNA3/CAT or JEVAX group.

TABLE 4. Ability of maternal antibody from JEV nucleic acid-vaccinated female mice to protect their pups from fatal JE

Vaccinated mother[a]		JEV-challenged pups			
Vaccine	$PRNT_{90}$	Age (days)	No. of survivors/ total in litter	Avg survival time (days)	ELISA[b]
1 × pCDJE2-7	40	4	0/11	5.27	
2 × pCDJE2-7	80	4	12/12	NA[c]	12/12
2 × JEVAX	20	3	0/16	4.75	
2 × pCDNA3/CAT	<10	5	0/14	4.00	
1 × pCDJE2-7	20	15	5/11	10.0	5/5
2 × pCDJE2-7	40	14	8/12	13.75	7/8
2 × JEVAX	80	13	5/5	NA	5/5
2 × pCDNA3/CAT	<10	14	0/14	6.14	

[a] Mice were inoculated i.m. with one or two 100-μg doses of pCDJE2-7 DNA or twice s.c. with one-fifth of the adult human dose of JEVAX. Serum samples were collected 9 weeks postvaccination for PRNT testing prior to mating with nonimmune male.

[b] Number of JEV ELISA antibody-positive animals (titer ≥ 1:400)/number of survivors. Serum specimens were collected for testing 12 weeks after challenge.

[c] NA, not applicable.

Passive protection of neonatal mice correlated with the maternal antibody titer. Female 3-week-old ICR mice were vaccinated with one or two doses of pCDJE2-7 plasmid DNA (100 μg/100 μl) or twice with one-fifth of the adult human dose of JEVAX. For evaluation of passive protection by maternal antibody, pups were obtained from matings of experimental females with nonimmunized male mice. Pups were challenged by the i.p. route at 3 to 5 or 13 to 15 days after birth with SA14 virus (5,000 PFU/100 μl). Survival rates and average survival time correlated with the maternal neutralizing antibody titers (Table 4). One hundred percent of pups nursed by mothers with a PRNT of 1:80 survived viral infection regardless of the type of vaccine received by the mothers. None of the pups from mothers which received pCDNA3/CAT plasmid DNA survived (Table 4). Partial protection (45% [5 of 11 pups] to 67% [8 of 12 pups]) was observed in older pups that were nursed by the mothers which had serum PRNT titers of 1:20 and 1:40, respectively. However, none of the 3-day-old pups survived virus challenge when the mothers had a serum PRNT titer of 1:20 or 1:40. Maternally transferred antibody can only be detected in the circulation of the young mouse up to 40 days after birth. An appreciable level of maternally derived antibody is maintained in the circulation of the young mouse 24 days or more postpartum (1). JEV ELISA antibody detected in the serum of 97% (29 of 30) of the postchallenge pups at 12 weeks after virus challenge was unlikely to be residual maternally transferred antibody. The presence of JEV antibody in the surviving pups challenged at 3 to 4 or 13 to 15 days of age strongly suggested that maternal antibody did not provide sterilizing immunity to the pups. It also indicated that 3- to 4- or 13- to 15-day-old mice could mount an immune reaction to a live-virus challenge. Partial protection in older pups could be explained by the opportunity to accumulate a large quantity of passive antibody due to the length of nursing time before challenge. One randomly selected postchallenge serum sample also reacted with prM and E proteins by Western blotting (Fig. 3).

DISCUSSION

The flavivirus virion contains a capsid protein (C), a membrane protein (M), and an E protein. The prM MAbs, exhibiting weak or undetectable neutralizing activity in vitro, can provide passive protection following DEN2 virus challenge (16). However, the E protein plays a dominant role in generating neutralizing antibodies and providing protective immunity in the host. Passive transfer of JEV E-specific neutralizing MAbs has been shown to protect recipients from JEV-induced fatal encephalitis (3, 16, 32, 55). Antigenic and structural analysis using various panels of MAbs has shown that most of the E protein epitopes that elicit virus-neutralizing antibodies are conformationally dependent (9, 40). Coexpression of both proteins as type I transmembrane proteins is essential to maintain proper E conformation and prevent the E protein from undergoing irreversible, low-pH-catalyzed conformational changes (8–10, 19, 50). A 2-kb genomic region, from the internal signal peptide at the carboxyl terminus of C to the transmembrane domain at the carboxyl terminus of the E gene, is essential for expressing authentic proteins. These authentic prM and E proteins are able to self-assemble into virus-like particles in cells infected by either recombinant vaccinia virus or alphavirus vector or in cells transformed by recombinant plasmid DNA (4, 19, 22, 48; Hunt and Chang, unpublished data).

A gene cassette including the elements listed above was amplified from SA14 virus by RT-PCR in the present study. Optimal sequence composition surrounding the translation initiation site (−9 to +4) was incorporated into the 14DV398 amplifying primer (2, 26, 27) (Fig. 1). Recombinant plasmids containing the CMV early gene promoter/enhancer and the BHG poly(A) terminator as transcription regulatory elements expressed JEV proteins with the highest efficiency in three different cell lines. Protein expression and the serological response of mice immunized with DNA vaccine were not influenced by the presence or absence of the SV40 ori or an intron sequence in recombinant plasmids. Virus-specific proteins, secreted into culture medium, could be detected by antigen capture ELISA as early as 48 h after plasmid transformation (data not shown). The authenticity of the E protein produced by the pCDJE2-7 stably transformed cell line, JE-4B, was demonstrated by MAb epitope mapping.

Vaccine potential and characteristics of various eukaryotic plasmids that express flavivirus prM and E proteins are summarized in Tables 5 and 6. All constructs listed had the same transcriptional control elements and similar viral gene cassettes. DEN2 plasmid, which contains prM and 91% of E, is the only exception (Table 6). The JEV DNA vaccine reported in this study is the only construct that stimulated complete protective immunity in mice by a single dose of vaccine given by the i.m. route (Table 5). Sequences surrounding the translation initiation site and the composition of the signal peptide preceding the prM protein are the two major differences among the constructs that may contribute to increasing the vaccine potential of our construct (Table 6). Conserved features of the sequences which flank vertebrate translation initiation sites include a strong preference for purine at the −3 position; a higher frequency of G at positions −9, −6, −3, and +4; and a preference for A or C at positions −5, −4, −2, and −1 (2). Instead of the sequence used in previous publications, the sequence used in our construct was −9 · GCCGCCGCC ATGG, which fits the general criteria listed above. Although less than 1% of eukaryotic mRNA sequences exhibit this sequence, the experimental data have suggested that this sequence provides exceptionally high levels of translation potential (2, 26).

Signal peptides determine translocation and orientation of inserted protein, hence the topology of prM and E. Signal peptide differences in our plasmid construct may account for the efficient translocation and correct topology, thus increasing prM and E secretion. A machine-learning program using neu-

TABLE 5. Vaccine potential of various eukaryotic plasmids that express flavivirus prM and E proteins[a]

Virus	In vitro secretion of EPs	Immunization			Protection from virus challenge	Reference
		Dosage	Route/method	Neutralizing antibody[b]		
JE	Yes	25–100 μg × 1	i.m./needle	Yes ($1:20–1:160_{90\%}$)	100%	This report
	ND	100 μg × 2	i.m./needle	No	Partial	30
	ND	10–100 μg × 2	i.m. or i.d./needle	Yes ($1:10–1:20_{90\%}$)	100%	24
MVE	Yes	100 μg × 4	i.m./needle	ND	Partial	4
	Yes	1–2 μg × 2–4	i.d./gene gun	Yes ($80–320_{50\%}$)	100%	4
SLE	ND	100 μg × 2	i.m./needle	No	Partial	38
CEE	ND	1 μg × 1–2	i.d./gene gun	Yes ($1:100–1:1{,}600_{80\%}$)	100%	49
RSSE	ND	1 μg × 1–2	i.d./gene gun	ND	100%	49
DEN2	ND	200 μg × 3	i.d./needle	Yes ($1:10–1:320_{50\%}$)	None	17

[a] MVE, Murray Valley encephalitis; CEE, Central European encephalitis; RSSE, Russian spring-summer encephalitis; i.d., intradermal; ND, not done.
[b] Plaque reduction neutralization titer followed by percentage reduction endpoint used in the test.

ral networks trained on eukaryotes (SignalP-NN at http://www.cbs.dtu.dk/services/) was applied to test the efficiency of the prM signal peptide sequence in the different plasmid constructs (34) (Table 6). The most probable location and orientation of transmembrane helices in the prM-E protein were then determined by a hidden Markov model-trained computer program (6 [TMHMM at http://www.cbs.dtu.dk/services/]). SignalP-NN searches correctly predicted the signal peptidase cleavage site of all constructs. However, a considerable difference in cleavage potential (C score, between 0.578 and 1.000) was observed (Table 6). Cleavage potential differences may be influenced by the amino acid composition and length of the h region in various constructs (44).

The TMHMM program correctly predicted five transmembrane helices encoded in the prM-E protein. Significant difference in the probable orientation of the first transmembrane helix was observed in three JEV constructs (Fig. 5). In our pCDJE2-7 construct, the first 12 amino acids of the n region form a short loop in the cytoplasmic side that causes the following h region (transmembrane helix) to be inserted in a tail orientation. Secretion of JEV protein could be detected by antigen capture ELISA in pCDJE2-7 transient expression studies in which less than 5% of the cells were positive by IFA (data not shown). Thus, there is a high probability that prM and E proteins expressed by pCDJE2-7 would be expressed in the correct orientation, as type I transmembrane proteins (Fig. 5A). There is also a high probability that the prM protein of pcDNA3JEME could be expressed as a type II membrane protein with its transmembrane h region inserted in a head orientation because of the absence of positively charged amino acids in its n region (Fig. 5B). Efficient protein synthesis in conjunction with correct topology of expressed prM and E (Fig. 5A) would most likely enhance EP formation and secretion in transformed cells.

Another characteristic that could explain the excellent vaccine potential of our JEV construct is its ability to produce EPs which have a virus-like polymeric structure that enhances antigenic stability and provides a high-density presentation to antigen-presenting cells, such as macrophages, dendritic cells, and Langerhans cells (5). When DNA is given by the i.m. route, the majority of antigen is expressed by non-antigen-presenting muscle cells. The efficacy of a DNA vaccine is therefore dependent on transfection of antigen-presenting cells or to reprocessing of antigen derived from other cells. Muscle cells transfected by our construct could conceivably synthesize and secrete EPs, which are highly immunogenic and have been shown to elicit good cellular and humoral responses (22, 23).

Genetic JEV vaccine that induced a completely protective immunity in neonatal mice and a maternally transferable protective immunity in young adult mice by a single i.m. immunization was demonstrated in this study. Additional studies are planned to address the effectiveness of a DNA vaccine in overcoming the potential influence of maternally transferred flavivirus antibodies on the induction of JEV antibody in neonatal mice.

Immunization of pigs is a theoretical means of interrupting transmission and amplification of JEV and thereby preventing human infections (43). The JEV DNA vaccine could also be used as a veterinary vaccine in pregnant sows to prevent JEV-induced stillbirth and abortion (51, 53). Maternally transferred antibody could also interrupt piglets as the JEV-amplifying host and thus reduce human infection.

TABLE 6. Characteristics of various eukaryotic plasmids expressing flavivirus prM and E proteins

Virus[a]	Plasmid	Sequence surrounding translation initiation site	Amino acids preceding prM protein[b]	SP potential (C score)[c]	Reference
JE	pCDJE2-7	−9•GCCGCCGCC**ATG**G•+4	MG**RK**QN**KR**GGNEGSIMWLASLAVVIACAGA /MKL	Yes (0.921)	This report
	pJME	−9•GGCTCAATC**ATG**G•+4	MWLASLAVVIACAGA /MKL	Yes (0.578)	30
	pCDNA3JEME	−9•GAATTCACC**ATG**G•+4	MNEGSIMWLASLAVVIACAGA /MKL	Yes (0.921)	24
MVE	pCDNA3.prM-E	−9•TGATTTCAA**ATG**T•+4	MS**KKR**GGSETSVLMVIFMLIGFAAA /LKL	Yes (0.819)	4
SLE	pSLE1	?	?LDTIN**RR**PS**KKR**GGT**R**SLLGLAALIGLASS /LQL	Yes (0.709)	38
DEN2	p1012D2ME	?	?AGMIIMLIPTVMA /FHL	Yes (0.646)	17
TBE	SV-PE_{wt}	−9•GCGGCCGCC**ATG**G•+4	MVGLQ**KR**G**KRR**SATDWMSWLLVITLLGMTLA /ATV	Yes (1.000)	48
RSSE	pWRG7077	−9•GTAGACAGG**ATG**G•+4	MGWLLVVVLLGVTLA /ATV	Yes (0.762)	50
CEE	pWRG7077	−9•ACGGACAGG**ATG**G•+4	MSWLLVITLLGMTIA /ATV	Yes (0.609)	50

[a] Abbreviations are as given in Table 5, footnote *a*.
[b] Single amino acid code. Positively charged amino acid is indicated by bold letter. Signal peptidase cleavage site is indicated by /.
[c] Cleavage potential of signal peptide (SP) predicted by SignalP-NN at http://www.cbs.dtu.dk/services (34).

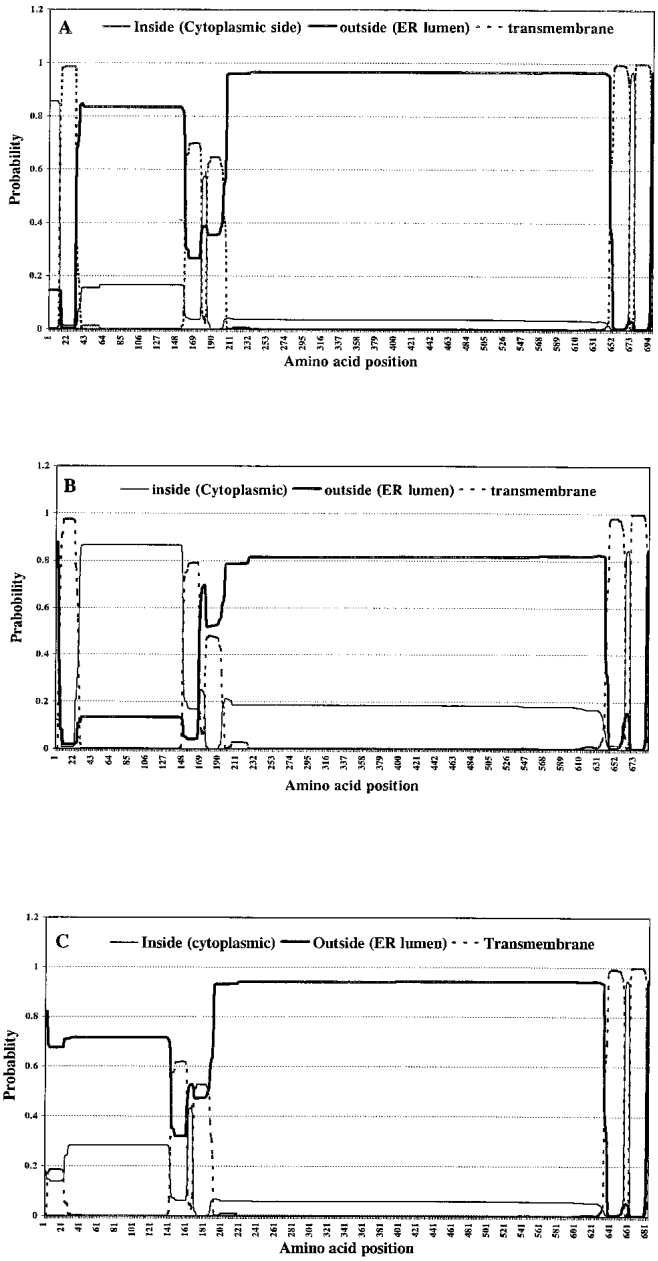


FIG. 5. Graphic representation, generated by the TMHMM program, indicating probable orientations of five transmembrane helices in the prM-E protein expressed by pCDJE2-7 (A), pcDNA3JEME (B), and pJME (C). ER, endoplasmic reticulum.

ACKNOWLEDGMENTS

We thank K. Yasui and M.-J. Zhang for providing JEV MAbs and J. Roehrig and B. Miller for useful discussion and advice. We thank D. Holmes, C. Lin, N. Frank, and T. Springfield for superb technical assistance and animal care.

REFERENCES

1. **Appleby, P., and D. Catty.** 1983. Transmission of immunoglobulin to foetal and neonatal mice. J. Reprod. Immunol. **5:**203–213.
2. **Cavener, D. R., and S. C. Ray.** 1991. Eukaryotic start and stop translation sites. Nucleic Acids Res. **19:**3185–3192.
3. **Cecilia, D., D. A. Gadkari, N. Kedarnath, and S. N. Ghosh.** 1988. Epitope mapping of Japanese encephalitis virus envelope protein using monoclonal antibodies against an Indian strain. J. Gen. Virol. **69:**2741–2747.
4. **Colombage, G., R. Hall, M. Pavy, and M. Lobigs.** 1998. DNA-based and alphavirus-vectored immunisation with prM and E proteins elicits long-lived and protective immunity against the flavivirus, Murray Valley encephalitis virus. Virology **250:**151–163.
5. **Condon, C., S. C. Watkins, C. M. Celluzzi, K. Thompson, and L. D. Falo, Jr.** 1996. DNA-based immunization by in vivo transfection of dendritic cells. Nat. Med. **2:**1122–1128.
6. **Erik, L. L., S. G. von Heijne, and A. Krogh.** 1998. A hidden Markov model for predicting transmembrane helices in protein sequences, p. 175–182. *In* J. Glasgow, T. Littlejohn, F. Major, R. Lathrop, D. Sankoff, and C. Sensen (ed.), Proceedings of the Sixth International Conference on Intelligent Systems for Molecular Biology. AAAI Press, Menlo Park, Calif.
7. **Gentry, M. K., E. A. Henchal, J. M. McCown, W. E. Brandt, and J. M. Dalrymple.** 1982. Identification of distinct antigenic determinants on dengue-2 virus using monoclonal antibodies. Am. J. Trop. Med. Hyg. **31:**548–555.
8. **Guirakhoo, F., R. A. Bolin, and J. T. Roehrig.** 1992. The Murray Valley encephalitis virus prM protein confers acid resistance to virus particles and alters the expression of epitopes within the R2 domain of E glycoprotein. Virology **191:**921–931.
9. **Heinz, F. X., R. Berger, W. Tuma, and C. Kunz.** 1983. A topological and functional model of epitopes on the structural glycoprotein of tick-borne encephalitis virus defined by monoclonal antibodies. Virology **126:**525–537.
10. **Heinz, F. X., K. Stiasny, G. Puschner-Auer, H. Holzmann, S. L. Allison, C. W. Mandl, and C. Kunz.** 1994. Structural changes and functional control of the tick-borne encephalitis virus glycoprotein E by the heterodimeric association with protein prM. Virology **198:**109–117.
11. **Hennessy, S., Z. Liu, T. F. Tsai, B. L. Strom, C. M. Wan, H. L. Liu, T. X. Wu, H. J. Yu, Q. M. Liu, N. Karabatsos, W. B. Bilker, and S. B. Halstead.** 1996. Effectiveness of live-attenuated Japanese encephalitis vaccine (SA14-14-2): a case-control study. Lancet **347:**1583–1586.
12. **Hunt, A. R., and C. H. Calisher.** 1979. Relationships of bunyamwera group viruses by neutralization. Am. J. Trop. Med. Hyg. **28:**740–749.
13. **Igarashi, A.** 1978. Isolation of a Singh's *Aedes albopictus* cell clone sensitive to dengue and Chikungunya viruses. J. Gen. Virol. **40:**531–544.
14. **Kaufman, B. M., P. L. Summers, D. R. Dubois, W. H. Cohen, M. K. Gentry, R. L. Timchak, D. S. Burke, and K. H. Eckels.** 1989. Monoclonal antibodies for dengue virus prM glycoprotein protect mice against lethal dengue infection. Am. J. Trop. Med. Hyg. **41:**576–580.
15. **Kimura-Kuroda, J., and K. Yasui.** 1983. Topographical analysis of antigenic determinants on envelope glycoprotein V3 (E) of Japanese encephalitis virus, using monoclonal antibodies. J. Virol. **45:**124–132.
16. **Kimura-Kuroda, J., and K. Yasui.** 1988. Protection of mice against Japanese encephalitis virus by passive administration with monoclonal antibodies. J. Immunol. **141:**3606–3610.
17. **Kochel, T., S. J. Wu, K. Raviprakash, P. Hobart, S. Hoffman, K. Porter, and C. Hayes.** 1997. Inoculation of plasmids expressing the dengue-2 envelope gene elicit neutralizing antibodies in mice. Vaccine **15:**547–552.
18. **Konishi, E., I. Kurane, P. W. Mason, R. E. Shope, N. Kanesa-Thasan, J. J. Smucny, C. H. Hoke, Jr., and F. A. Ennis.** 1998. Induction of Japanese encephalitis virus-specific cytotoxic T lymphocytes in humans by poxvirus-based JE vaccine candidates. Vaccine **16:**842–849.
19. **Konishi, E., and P. W. Mason.** 1993. Proper maturation of the Japanese encephalitis virus envelope glycoprotein requires cosynthesis with the premembrane protein. J. Virol. **67:**1672–1675.
20. **Konishi, E., S. Pincus, B. A. Fonseca, R. E. Shope, E. Paoletti, and P. W. Mason.** 1991. Comparison of protective immunity elicited by recombinant vaccinia viruses that synthesize E or NS1 of Japanese encephalitis virus. Virology **185:**401–410.
21. **Konishi, E., S. Pincus, E. Paoletti, W. W. Laegreid, R. E. Shope, and P. W. Mason.** 1992. A highly attenuated host range-restricted vaccinia virus strain, NYVAC, encoding the prM, E, and NS1 genes of Japanese encephalitis virus prevents JEV viremia in swine. Virology **190:**454–458.
22. **Konishi, E., S. Pincus, E. Paoletti, R. E. Shope, T. Burrage, and P. W. Mason.** 1992. Mice immunized with a subviral particle containing the Japanese encephalitis virus prM/M and E proteins are protected from lethal JEV infection. Virology **188:**714–720.
23. **Konishi, E., K. S. Win, I. Kurane, P. W. Mason, R. E. Shope, and F. A. Ennis.** 1997. Particulate vaccine candidate for Japanese encephalitis induces long-lasting virus-specific memory T lymphocytes in mice. Vaccine **15:**281–286.
24. **Konishi, E., M. Yamaoka, Khin-Sane-Win, I. Kurane, and P. W. Mason.** 1998. Induction of protective immunity against Japanese encephalitis in mice by immunization with a plasmid encoding Japanese encephalitis virus premembrane and envelope genes. J. Virol. **72:**4925–4930.
25. **Kozak, M.** 1984. Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. Nucleic Acids Res. **12:**857–872.
26. **Kozak, M.** 1987. At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. J. Mol. Biol. **196:**947–950.
27. **Kozak, M.** 1997. Recognition of AUG and alternative initiator codons is augmented by G in position +4 but is not generally affected by the nucleotides in positions +5 and +6. EMBO J. **16:**2482–2492.
28. **Ku, C. C., C. C. King, C. Y. Lin, H. C. Hsu, L. Y. Chen, Y. Y. Yueh, and G. J. Chang.** 1994. Homologous and heterologous neutralization antibody re-

sponses after immunization with Japanese encephalitis vaccine among Taiwan children. J. Med. Virol. **44:**122–131.
29. **Kuno, G., G. J. Chang, K. R. Tsuchiya, N. Karabatsos, and C. B. Cropp.** 1998. Phylogeny of the genus *Flavivirus*. J. Virol. **72:**73–83.
30. **Lin, Y. L., L. K. Chen, C. L. Liao, C. T. Yeh, S. H. Ma, J. L. Chen, Y. L. Huang, S. S. Chen, and H. Y. Chiang.** 1998. DNA immunization with Japanese encephalitis virus nonstructural protein NS1 elicits protective immunity in mice. J. Virol. **72:**191–200.
31. **Liu, Z. L., S. Hennessy, B. L. Strom, T. F. Tsai, C. M. Wan, S. C. Tang, C. F. Xiang, W. B. Bilker, X. P. Pan, Y. J. Yao, Z. W. Xu, and S. B. Halstead.** 1997. Short-term safety of live attenuated Japanese encephalitis vaccine (SA14-14-2): results of a randomized trial with 26,239 subjects. J. Infect. Dis. **176:**1366–1369.
32. **Mason, P. W., J. M. Dalrymple, M. K. Gentry, J. M. McCown, C. H. Hoke, D. S. Burke, M. J. Fournier, and T. L. Mason.** 1989. Molecular characterization of a neutralizing domain of the Japanese encephalitis virus structural glycoprotein. J. Gen. Virol. **70:**2037–2049.
33. **McCown, J., M. Cochran, R. Putnak, R. Feighny, J. Burrous, E. Henchal, and C. Hoke.** 1990. Protection of mice against lethal Japanese encephalitis with a recombinant baculovirus vaccine. Am. J. Trop. Med. Hyg. **42:**491–499.
34. **Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heijne.** 1997. A neural network method for identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Int. J. Neural Syst. **8:**581–599.
35. **Nitayaphan, S., J. A. Grant, G. J. Chang, and D. W. Trent.** 1990. Nucleotide sequence of the virulent SA-14 strain of Japanese encephalitis virus and its attenuated vaccine derivative, SA-14-14-2. Virology **177:**541–552.
36. **Nothdurft, H. D., T. Jelinek, A. Marschang, H. Maiwald, A. Kapaun, and T. Loscher.** 1996. Adverse reactions to Japanese encephalitis vaccine in travellers. J. Infect. **32:**119–122.
37. **Obijeski, J. F., D. H. Bishop, E. L. Palmer, and F. A. Murphy.** 1976. Segmented genome and nucleocapsid of La Crosse virus. J. Virol. **20:**664–675.
38. **Phillpotts, R. J., K. Venugopal, and T. Brooks.** 1996. Immunisation with DNA polynucleotides protects mice against lethal challenge with St. Louis encephalitis virus. Arch. Virol. **141:**743–749.
39. **Poland, J. D., C. B. Cropp, R. B. Craven, and T. P. Monath.** 1990. Evaluation of the potency and safety of inactivated Japanese encephalitis vaccine in US inhabitants. J. Infect. Dis. **161:**878–882.
40. **Roehrig, J. T., R. A. Bolin, and R. G. Kelly.** 1998. Monoclonal antibody mapping of the envelope glycoprotein of the dengue 2 virus, Jamaica. Virology **246:**317–328.
41. **Roehrig, J. T., A. R. Hunt, A. J. Johnson, and R. A. Hawkes.** 1989. Synthetic peptides derived from the deduced amino acid sequence of the E-glycoprotein of Murray Valley encephalitis virus elicit antiviral antibody. Virology **171:**49–60.
42. **Roehrig, J. T., J. H. Mathews, and D. W. Trent.** 1983. Identification of epitopes on the E glycoprotein of Saint Louis encephalitis virus using monoclonal antibodies. Virology **128:**118–126.
43. **Rosen, L.** 1986. The natural history of Japanese encephalitis virus. Annu. Rev. Microbiol. **40:**395–414.
44. **Sakaguchi, M., R. Tomiyoshi, T. Kuroiwa, K. Mihara, and T. Omura.** 1992. Functions of signal and signal-anchor sequences are determined by the balance between the hydrophobic segment and the N-terminal charge. Proc. Natl. Acad. Sci. USA **89:**16–19.
45. **Sakaguchi, M., M. Yoshida, W. Kuroda, O. Harayama, Y. Matsunaga, and S. Inouye.** 1997. Systemic immediate-type reactions to gelatin included in Japanese encephalitis vaccines. Vaccine **15:**121–122.
46. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
47. **Sato, T., C. Takamura, A. Yasuda, M. Miyamoto, K. Kamogawa, and K. Yasui.** 1993. High-level expression of the Japanese encephalitis virus E protein by recombinant vaccinia virus and enhancement of its extracellular release by the NS3 gene product. Virology **192:**483–490.
48. **Schalich, J., S. L. Allison, K. Stiasny, C. W. Mandl, C. Kunz, and F. X. Heinz.** 1996. Recombinant subviral particles from tick-borne encephalitis virus are fusogenic and provide a model system for studying flavivirus envelope glycoprotein functions. J. Virol. **70:**4549–4557.
49. **Schmaljohn, C., L. Vanderzanden, M. Bray, D. Custer, B. Meyer, D. Li, C. Rossi, D. Fuller, J. Fuller, J. Haynes, and J. Huggins.** 1997. Naked DNA vaccines expressing the prM and E genes of Russian spring summer encephalitis virus and Central European encephalitis virus protect mice from homologous and heterologous challenge. J. Virol. **71:**9563–9569.
50. **Stocks, C. E., and M. Lobigs.** 1998. Signal peptidase cleavage at the flavivirus C-prM junction: dependence on the viral NS2B-3 protease for efficient processing requires determinants in C, the signal peptide, and prM. J. Virol. **72:**2141–2149.
51. **Takashima, I., T. Watanabe, N. Ouchi, and N. Hashimoto.** 1988. Ecological studies of Japanese encephalitis virus in Hokkaido: interepidemic outbreaks of swine abortion and evidence for the virus to overwinter locally. Am. J. Trop. Med. Hyg. **38:**420–427.
52. **Tsai, T. F., G. J. Chang, and Y. X. Yu.** 1999. Japanese encephalitis vaccines, p. 672–710. *In* A. P. Stanley and W. A. Orenstein (ed.), Vaccines, 3rd ed. W. B. Saunders, Philadelphia, Pa.
53. **Ueba, N., T. Kimura, S. Nakajima, T. Kurimura, and T. Kitaura.** 1978. Field experiments on live attenuated Japanese encephalitis virus vaccine for swine. Biken J. **21:**95–103.
54. **Yasuda, A., J. Kimura-Kuroda, M. Ogimoto, M. Miyamoto, T. Sata, T. Sato, C. Takamura, T. Kurata, A. Kojima, and K. Yasui.** 1990. Induction of protective immunity in animals vaccinated with recombinant vaccinia viruses that express PreM and E glycoproteins of Japanese encephalitis virus. J. Virol. **64:**2788–2795.
55. **Zhang, M. J., M. J. Wang, S. Z. Jiang, and W. Y. Ma.** 1989. Passive protection of mice, goats, and monkeys against Japanese encephalitis with monoclonal antibodies. J. Med. Virol. **29:**133–138.

Flavivirus DNA Vaccines

Current Status and Potential

GWONG-JEN J. CHANG, BRENT S. DAVIS, ANN R. HUNT, DEREK A. HOLMES, AND GORO KUNO

Division of Vector-Borne Infectious Diseases,
Centers for Disease Control and Prevention, Public Health Service,
United States Department of Health and Human Services,
Fort Collins, Colorado 80522, USA

ABSTRACT: The use of DNA-based vaccines is a novel and promising immunization approach for the development of flavivirus vaccines. This approach has been attempted in vaccine development for various virus species, including St. Louis encephalitis, Russian spring-summer encephalitis, Central European encephalitis, dengue serotypes 1 and 2, Murray Valley encephalitis, Japanese encephalitis, and West Nile viruses. However, very little is known about the factors affecting its efficacy. Recently, we demonstrated that a single intramuscular immunization of DNA vaccine of Japanese encephalitis and West Nile viruses protected mice and horses from virus challenge. Administration of these recombinant plasmid vectors resulted in endogenous expression and secretion of extracellular virus-like particles that correlated well with the induction of protective immunity. These results provided evidence that the virus-like particles composed of premembrane/membrane and envelope proteins are essential for eliciting immune responses similar to those induced by live, attenuated virus vaccines. The biosynthesis and protein processing of premembrane/membrane and envelope proteins that preserve the native conformation and glycosylation profiles identical to virion proteins could be determined by the effectiveness of the transmembrane signal sequence located at the amino-terminus of premembrane protein. The use of DNA vaccines in multivalent and/or combination vaccines designed to immunize against multiple flaviviruses is also a promising area of development.

KEYWORDS: Flavivirus; vaccine; infection; protein

INTRODUCTION

Epidemics of flavivirus infections continue to be a major public health concern worldwide. The yellow fever (YF) virus is the prototype member of the genus *Flavivirus* that includes 70 distinct virus species.[1] More than two billion people are at risk of being infected with members of this group of viruses, including Japanese encephalitis (JE) virus in Asia and Australia; YF virus in Africa and Latin America; West

Address for correspondence: G-J. J. Chang, Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service, U.S. Department of Health and Human Services, P.O. Box 2087, Fort Collins, CO 80522-2087. Voice: 970-221-6497; fax: 970-221-6476.
gxc7@cdc.gov

Nile (WN) virus in Africa, Central Europe, and America; tick-borne encephalitis (TBE) complex viruses, including Russian spring-summer encephalitis (RSSE) and Central European encephalitis (CEE) viruses in the temporal regions of Europe and Asia; and four serotypes of dengue (DEN-1, -2, -3, and -4) viruses in the tropical and subtropical regions of the world. Conventional vaccines have contributed enormously to the improvement of human health, yet infectious diseases remain the leading cause of mortality worldwide. With the exceptions of YF, JE, and TBE, there is no vaccine available to prevent other flavivirus infection in humans or animals.

The YF 17D vaccine, available since 1936, has been widely regarded as one of the safest and most effective arboviral vaccine ever developed. However, even such a vaccine has not succeeded in controlling the outbreak of the disease. Some of the problems were the failure to implement a routine vaccination program for financial and other reasons, and inadequate public infrastructure to support the deployment of vaccine in developing countries. Significant efforts have been made to develop new and effective vaccine strategies to combat flavivirus infection in humans as well as in domestic animals.[2,3] The reverse genetic technique using infectious cDNA clones derived from 17D, PDK-53 (an attenuated DEN-2 vaccine virus) and genetic-engineered, attenuated DEN-4 814669 strain, has greatly increased the potential of creating the genetic-engineered, live-attenuated chimeric flavivirus vaccine.[4–10] However, there is a potential risk of serious infection by virulent viruses that may arise by gene reversion or recombination derived from attenuated vaccine viruses, as demonstrated by the problem encountered in the polio virus eradication campaign and in vaccine development for HIV vaccine.[11–13] With infectious clone technology, it is still difficult to formulate a multivalent live-attenuated vaccine regimen due to potential homologous or heterologous interference during virus replication.

Inoculation of animals with purified plasmid vectors (DNA) represents a novel means of expressing antigens *in vivo* for the generation of both humoral and cellular responses that protect against infectious disease agents and prevent illness due to cancer and autoimmune diseases.[14,15] Plasmid DNA is stable at ambient temperature and amenable to developing a standardized generic manufacturing process. It allows a rapid screening and manipulation of gene sequence to identify and enhance vaccine potential. Plasmid DNA vaccines thus provide an invaluable alternative to attenuated, inactivated, or viral-vectored subunit vaccines.

Members of the genus *Flavivirus* have a positive-sense single-strand RNA genome approximately 11 kb in size, which encodes for a single polyprotein precursor arranged in the order of capsid (C), premembrane (prM), envelope (E), and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5).[16,17] Processing of the N-terminal polyprotein by the host signalase and virus serine protease complex, NS2B/NS3, yields three structural proteins (C, prM, and E) that are assembled into the virion. Virus assembly occurs at the membrane of the endoplasmic reticulum (ER) and leads to formation of immature virions containing prM. The immature virions are then transported from the ER to the Golgi apparatus where the majority of prM is cleaved to M by the furin-like host protease during exocytosis.[18] The immature virions do not exhibit low-pH-induced conformational changes or fusion activity in the low-pH exocytic vesicles. Thus, the significant function of prM is to prevent irreversible conformational changes in the E protein that leads to inactivation of the virus. Some DEN virus prM-specific monoclonal

antibodies have a detectable virus-neutralizing activity and are protective in mice.[19] However, E protein is believed to mediate receptor binding and membrane fusion; further, it induces a protective immunity. The importance of antibodies to E protein in antiviral protection has been demonstrated in passive transfer experiments.[20–23] In addition to infectious virions, noninfectious virus-like particles (VLPs) that contain the prM/M and E proteins, but lack the nucleocapsid, are released during virus infection. Mason and coworkers have demonstrated that similar particles can be obtained in secreted form when prM and E proteins of JE virus are coexpressed properly in the absence of the C protein and suggested that the VLPs represent capsidless empty viral envelopes.[24,25] Thus, the attention has been focused on the construction of flavivirus DNA vaccine that has a potential of expressing VLPs composed of prM and E proteins.

Flavivirus DNA vaccines for SLE,[26] RSSE and CEE,[27,28] DEN-1,[29] DEN-2,[30] JE,[31–33] MVE,[34] WN,[35] and louping ill (LI)[36] viruses have been developed. All these plasmid DNA constructs contained similar transcriptional regulators and flavivirus gene cassette, induced antibody responses, and provided full or partial protection from virus challenge in mice. In this report, we summarize the current status of flavivirus DNA vaccines and then attempt to correlate the immunogenicity and protective efficacy of various DNA vaccines with genetic constituents of the transcriptional unit. Finally, we discuss the future direction for flavivirus DNA vaccine development.

CURRENT STATUS OF FLAVIVIRUS DNA VACCINES

Expression of the gene cassette in the recombinant DNA vaccine is regulated by transcriptional and translational regulators that include the following basic components: promoter/enhancer, translation initiation sequence, translation terminator, and transcription terminator/polyadenylation signal. The characteristics of the transcriptional and translational control elements in the plasmids expressing flavivirus prM and E proteins are summarized in TABLE 1. The most commonly used transcriptional regulator is human cytomegalovirus immediate-early gene promoter (CMV IE) used in conjunction with bovine growth hormone transcription terminator/polyadenylation sequence [BGH/poly (A)]. The SV40 early promoter used in conjunction with SA40 poly (A) in the SV-PE plasmid has been used to express TBE prM and E proteins with great efficiency.[37] Expression of many genes may depend on or increase by the inclusion of an intron sequence, located at the 5′-nontranslational region of transcribed mRNA. Some of the vaccine plasmids also contain an intron sequence (TABLE 1). Experiments conducted in our lab indicated that the optimal plasmid for *in vitro* JE virus prM-E expression utilizes the CMV IE and the BGH/poly (A).[33] In addition, we demonstrated that inclusion of an intron sequence or a eukaryotic replication origin, $SV40_{ori}$, in the plasmid with CMV IE and BGH/poly (A) neither increases *in vitro* antigen expression nor enhances protective immune response.

In general, vaccine potential, measured by induction of neutralizing (Nt) antibody and protective efficacy after virus challenge, can be improved by multiple intramuscular (i.m.), intradermal (i.d.), or "gene gun" (g.g.) deliveries of DNA vaccine. The most common method is i.m. injection by which plasmid DNA, formulated in

TABLE 1. Characteristics of transcriptional and translational control elements in various eukaryotic plasmids expressing flavivirus prM and E protein

Plasmid	Virus	Promoter	Intron	Kozak sequence surrounding translation initiation site	Poly (A)	Eukaryotic origin of replication	Reference
pSLE1	SLE	CMV IE	No	?	CMV IE	No	26
pJME	JE	CMV IE	No	–9•CGGCTCAATC**ATG**GC•+4	BGH	$SV40_{ori}$	31
pCJEME	JE	CMV IE	No	–9•CGAATTCACC**ATG**GC•+4	BGH	$SV40_{ori}$	32
pNJEME	JE	CMV IE	Yes	–9•CGAATTCACC**ATG**GC•+4	BGH	No	43
pCDJE2-7	JE	CMV IE	No	–9•CGCCGCCGCC**ATG**GC•+4	BGH	$SV40_{ori}$	33
pCBJE1-14	JE	CMV IE	No	–9•CGCCGCCGCC**ATG**GC•+4	BGH	No	33
pCIBJES14	JE	CMV IE	Yes	–9•CGCCGCCGCC**ATG**GC•+4	BGH	No	33
pCEJE	JE	CMV IE	No	–9•CGCCGCCGCC**ATG**GC•+4	SV40	OriP	33
pREJE	JE	RSV	No	–9•CGCCGCCGCC**ATG**GC•+4	SV40	OriP	33
pRCJE	JE	RSV	No	–9•CGCCGCCGCC**ATG**GC•+4	BGH	$SV40_{ori}$	33
pcDNA3 prM-E	MVE	CMV IE	No	–9•CTGATTTCAA**ATG**TC•+4	BGH	$SV40_{ori}$	34
pCBWN	WN	CMV IE	No	–9•CGCCGCCGCC**ATG**GC•+4	BGH	No	35
p1012D2ME	DEN-2	CMV IE	Yes	?	BGH	No	30
SV-PE	TBE	SV40	No	–9•CGCGGCCGCC**ATG**GC•+4	SV40	$SV40_{ori}$	37
		CMV	No	–9•CGCGGCCGCC**ATG**GC•+4	BGH	No	28
pWRG7077-RSSE	RSSE	CMV IE	Yes	–9•CGTAGACAGG**ATG**GC•+4	BGH	No	27
pWRG7077-CEE	CEE	CMV IE	Yes	–9•CACGGACAGG**ATG**GC•+4	BGH	No	27
pBK-prME	LI	CMV IE	No	–9•CATCCGCACC**ATG**AC•+4	BGH	No	36

NOTE: SLE, St. Louis encephalitis virus; JE, Japanese encephalitis virus; MVE, Murray Valley encephalitis virus; WN, West Nile virus; DEN-2, dengue serotype 2 virus; TBE, tick-borne encephalitis complex viruses, including Russian spring-summer encephalitis virus (RSSE) and Central European encephalitis virus (CEE); Li, louping ill virus; CMV, cytomegalovirus; RSV, respiratory syncytial virus; BGH, bovine growth hormone; $SV40_{ori}$, replication origin of SV40 virus; OriP, replication origin of human papillomavirus.

phosphate-buffered saline, is taken up by muscle cells in which the genes are expressed. Subsequent transfer of expressed gene product(s) to the antigen-presenting cells stimulates proper immune responses. In the administration by g.g., DNA-coated gold particles are propelled into skin by a high-pressure device to deliver DNA to the epidermis.[38] The injected gene is expressed by the specialized antigen-presenting cells, Langerhans' cells, capable of presenting transfected antigens to the T-helper component of the immune system. The amount of DNA-coated gold particles that can be administered in a single application is limited to 2.5 μg per 1 mg of gold beads. TBE DNA vaccine trials in monkeys indicated that between 3 and 12 applications per monkey may be required to achieve an effective vaccination.[39] One study using a WN virus DNA construct demonstrated that the i.m. electrotransfer method greatly increased the vaccine efficacy in mice.[35,40]

The first flavivirus DNA vaccine demonstrating partial protection against challenge virus in mice was the plasmid DNA encoding the prM and E genes of SLE virus.[26] In the study, control mice exhibited about 25% survival, but no Nt antibody was detected in the mice immunized with double doses of the vaccine. In the mice that received three i.d. injections of a recombinant DEN-2 plasmid DNA containing prM and 92% of the E gene, all mice developed anti-DEN-2 Nt antibody.[30] However, experiments using a two-dose schedule failed to protect mice against a lethal DEN-2 virus challenge. Incorporation of the immunostimulatory CpG motif containing pUC19 plasmid in the vaccine regimen improved antibody response to the DEN-2 vaccine.[41] Sixty percent of the mice immunized with DEN-2 DNA vaccine plus pUC19 survived the challenge compared with only 10% in the control group. In other experiments, BALB/C mice inoculated i.m. with 100 μg of pcD2ME two or three times at intervals of two weeks developed a low level of Nt antibody; however, strong anamnestic responses were observed on days 4 and 8 after challenge.[42]

The three DNA vaccines each designed on a different strain of JE virus showed different characteristics of stimulating Nt antibody and of protective efficacy after virus challenge.[31–33] The most promising result among three JE studies demonstrated that a single i.m. injection of a recombinant JE virus DNA induces a long-lasting protective immunity and that the recombinant DNA vaccine is as effective as the inactivated JE vaccine currently used for humans (JE-VAX) in preventing JE in mice.[33] Another study extended the observation that JE virus DNA vaccine is more effective in inducing long-lasting Nt antibody than the licensed vaccine in swine.[43] Similarly, a single i.m. injection of a WN virus DNA construct also induced Nt antibody and provided protection in mice and horses.[35]

The quality of immune responses obtained with DNA vaccination is determined by the molecular configuration and properties of the expressed antigen that govern whether the antigen is secreted by the cell, remains bound in the cell membrane, or remains sequestered within the cell.[44] The DNA construct that expressed a secreted particular form of the prM-E antigen of TBE is far superior to the constructs that synthesize intracellular or soluble secreted forms of the same antigen in terms of the extent and functionality of antibody responses and protection against virus challenge.[28] Furthermore, this study revealed that induction of a Th1 and/or Th2 response is dependent on the route of immunization (i.m. vs. g.g.) and that it is strongly influenced by the physical properties of the antigen. The plasmid expressing the VLP is partially able to overcome the imbalance in favor of Th2 immune response that is

inherent in g.g. immunization by stimulating both Th1 and Th2 immunity. LI virus DNA vaccine is not as effective as the recombinant Semliki Forest virus construct in inducing protective response in mice.[36] However, other investigators demonstrated that the g.g. route of DNA vaccination for tick-borne viruses (RSSE and CEE) not only induced protective Nt antibody, but also provided a sterile immunity in mice and monkeys.[27,39]

PARAMETERS AFFECTING GENE EXPRESSIONAND VACCINE POTENTIAL OF FLAVIVIRUS DNA VACCINE

Vaccine potential and characteristics of various eukaryotic plasmids that express flavivirus prM and E proteins are summarized in TABLES 1 and 2. Nearly all constructs listed have the same transcriptional control elements and a similar viral gene cassette, with the exception of the DEN-2 plasmid, p1012D2ME, which contains prM gene and 91% of the E gene (TABLE 1). Sequences surrounding the translation initiation site and the composition of the signal peptide preceding the prM protein are the two major differences among the constructs. These differences may contribute to the differences in quantity and quality of the protein synthesized and hence vaccine efficacy. Conserved features of the Kozak sequence flanking vertebrate translation initiation sites include a strong preference for purine at the −3 position; a higher frequency of G at positions −9, −6, −3, and +4; and a preference for A or C at positions −5, −4, −2, and −1.[45] The sequence used in our JE and WN virus constructs was −9•GCCGCCGCCATGG•+4, which fits the general criteria listed above.[33,35] Although less than 1% of eukaryotic mRNA sequences exhibit this sequence, the experimental data have suggested that this sequence provides exceptionally high levels of translation potential.[45,46] In the cell lines (COS-1) transformed by pCBJE1-14 or pCBWN plasmid, secretion of prM/M and E proteins to the culture media was as efficient as in virus infection (G-J. Chang, unpublished observation). Another DNA construct fitting Kozak's general consensus sequence is TBE virus for which the NotI restriction endonuclease with recognition sequence GCGGCCGC was used to construct SV-PE plasmid.[28,47] The cell line (COS-7) transformed with SV-PE plasmid also secreted the prM and E proteins in the form of VLP to the culture medium. One other observation further supported that a single base difference in the −3 position (G to C change) abrogated the target cell recognition because of reduced surface expression of a minigene-encoded lymphocytic choriomeningitis virus T cell epitope.[48] Thus, a proper Kozak sequence is crucial for efficient recombinant protein synthesis.

Signal peptides determine translocation and orientation of inserted protein—hence, the topology of prM and E proteins. The most common feature of signal peptides of eukaryotes consists of an 8–12 stretch of hydrophobic amino acids called the h-region.[49] The region between the initiator Met and the h-region, the n-region, usually has 1–5 amino acids and normally carries positively charged amino acids. Between the h-region and the cleavage site is the c-region, which consists of 3–7 polar, but mostly uncharged, amino acid residues. During viral polyprotein synthesis, modulation of the signalase cleavage site from a cryptic to cleavable conformation at the junction of C and prM proteins depends on prior removal of the C protein by

TABLE 2. Characteristics of the signal peptides and their vaccine of potentials among flavivirus DNA vaccine constructs

Plasmid	Signal peptide sequence preceding prM protein	Signal peptide probability[a]			Immunization protocol/protection
		SP	AP	C site	
pSLE1	?L**D**TIN**RR**PS**KKR**GGT**R**SLLGLAALIGLASS/LQLLSTYQG	0.702	0.292	0.352	i.m. × 2/partial
pJME	MWLASLAVVIACAGA/M**K**LSNFQG**K**	0.998	0.000	0.778	i.m. × 2/partial
pCJEME	MN**E**GSIMWLASLAVVIACAGA/M**K**LSNFQG**K**	0.985	0.012	0.785	i.m. × 2/100%
pCBJE1-14	MG**RK**QN**KR**GGN**E**GSIMWLASLAVVIACAGA/M**K**LSNFQG**K**	0.791	0.199	0.623	i.m. × 1/100%
pcDNA3 prM-E	MS**KKR**GGS**E**TSVLMVIFMLIGFAAA/L**K**LSNFQG**K**	0.721	0.277	0.622	i.m. × 4/partial g.g. × 2–4/100%
pCBWN	MG**KR**SAGSIMWLASLAVVIACAGA/VTLSNFQG**K**	0.976	0.024	0.526	i.m. × 1/100%
p1012D2ME	MNVL**R**GF**RKE**IG**R**MLNILN**RRRR**TAGMIIMLIPTVMA/F**H**LTT**R**NG**E**	0.165	0.778	0.164	i.d. × 2/none
SV-PE	MVGLQ**KR**G**KRR**SATDWMSWLLVITLLGMTLA/ATV**RKER**GD	0.943	0.056	0.899	i.m. or g.g. × 2/100%
pWRG7077-RSSE	MGWLLVVVLLGVTLA/ATV**RKER**GD	1.000	0.000	0.912	g.g. × 2/100%
pWRG7077-CEE	MSWLLVITLLGMTIA/ATV**RKER**GD	0.999	0.000	0.821	g.g. × 2/100%

NOTE: Single amino acid codes are used, and charged amino acids are highlighted by underlined bold letters. The signalase cleavage site separating SP and prM is indicated by a slash. DNA vaccines were inoculated by the intramuscular (i.m.), intradermal (i.d.), or gene gun (g.g.) method.

[a]The SignalP-HMM program (http://www.cbs.dtu.dk/services/SignalP-2.0/)[51] was applied to calculate the signal peptide (SP), anchor peptide (AP), and signalase cleavage site (C site) probabilities.

the viral protease complex, NS2B/NS3.[50] Therefore, it is critical to consider the effectiveness of the viral signal sequence when prM and E proteins are to be expressed alone by an expression plasmid.

Signal peptide differences in various plasmid constructs may account for the difference in protein translocation, cleavage site presentation, and correct topology—thus, prM and E secretion and VLP formation. A machine-learning computer program using a hidden Markov model (HMM) trained on eukaryotes (http://www.cbs.dtu.dk/services/SignalP-2.0/)[51] was applied to calculate the signal peptide probability of the prM signal peptide sequences in different plasmid constructs (TABLE 2). SignalP-HMM searches correctly predicted the signal peptidase cleavage sites in all constructs. However, considerable differences in cleavage probability (ranging between 0.164 and 1.000) and in signal peptide probability (ranging between 0.165 and 1.00) were observed (TABLE 2). The cleavage site and signal peptide probabilities are influenced by the positively charged amino acids in the n-region, the length of the hydrophobic amino acids in the h-region, and amino acid composition in the c-region in the constructs (FIG. 1).[52] Three JE virus plasmid constructs each derived from a different strain of JE virus showed different vaccine potentials.[31–33] The signal peptide sequences in these constructs are different in the length of n-region that may or may not contain charged amino acids (TABLE 2). The n-region containing positively charged amino acids forms a short loop in the cytoplasmic side that causes the h-region (transmembrane helix) to be inserted in a tail orientation, exposing the signalase cleavage site. In our study, secreted VLPs containing prM/M and E proteins could be purified from culture medium of the pCDJE2-7 transformed cell line, JE4B, or the pCBJE1-14 transiently transformed COS-1 cells. The gradient-purified VLPs and virions have identical immunological and biochemical properties. Processing efficiency from prM to mature M protein, the hallmark of flavivirus morphogenesis, is also similar between VLPs and virion particles.[53] Thus, there exists a high probability that prM and E proteins expressed by pCDJE2-7 and pCBJE1-14 would be expressed as type I transmembrane proteins in the orientation similar to that of virion prM and E.[33] On the other hand, the prM protein of pcDNA3JEME could be expressed as a type II membrane protein with its transmembrane h-region inserted in a head orientation because of the absence of positively charged amino acids in its n-region.[32] Efficient protein synthesis in conjunction with correct topology of expressed prM and E would most likely enhance VLP formation and secretion, thus promoting the immunogenicity of the DNA vaccine.[33]

We have taken advantage of the predictive power of the SignalP-HMM program and applied it to design the WN virus expression plasmid (TABLE 2).[35] The pCBWN plasmid consists of a short version of JE virus signal peptide followed by WN virus prM-E gene sequence. Vaccine potential of this construct was amply demonstrated

---

FIGURE 1. Graphic presentation of the signal peptide probabilities located at the N-terminus of the prM gene of the flavivirus DNA vaccine constructs. Each *panel* represents the graph generated by the SignalP-HMM program (http://www.cbs.dtu.dk/services/SignalP-2.0/)[51] using the signal peptide sequence encoded in each DNA construct as indicated in TABLE 2. The n-, h-, and c-region of a signal peptide are represented in *panel* A by ***n***, ***h***, and ***c***, respectively. *Panels* F and G, representing pSLE1 and p1012D2ME constructs, respectively, predict that the signal peptides in these two plasmids may contain a cryptic cleavage.

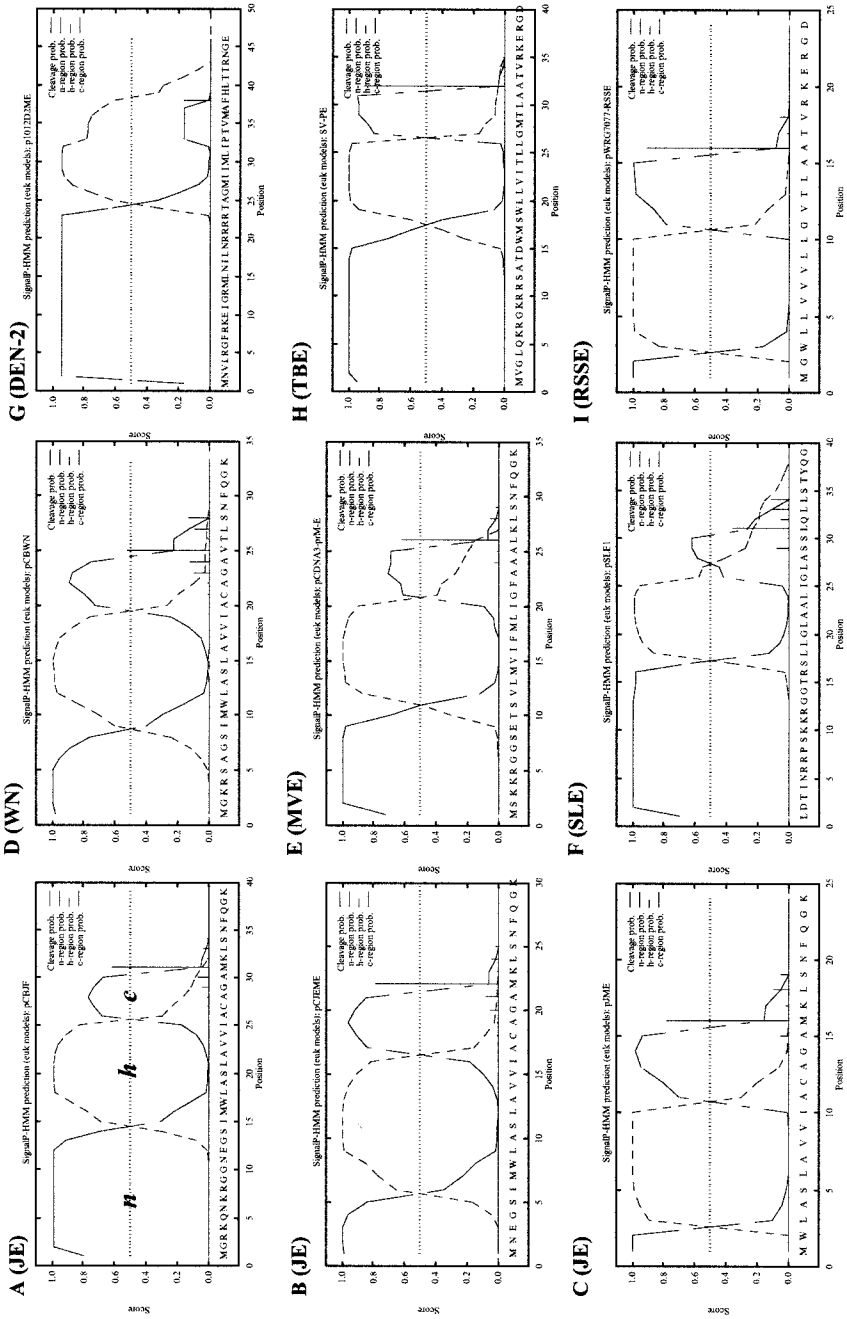


FIGURE 1. *See previous page for legend.*

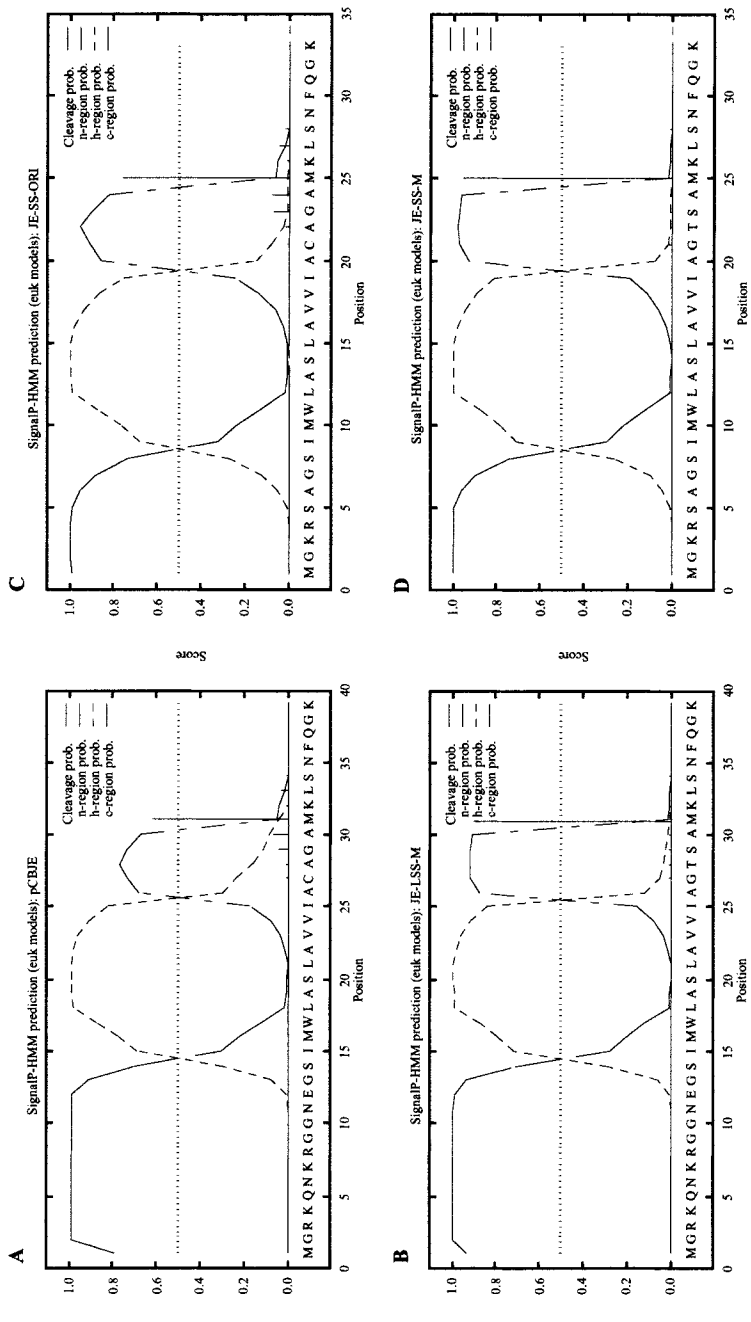


FIGURE 2. Signal peptide probability of the pCBJE1-14 (pCBJE) predicted by the SignalP-HMM program (*panel* A). The signal peptide probability can be improved by altering the c-region sequence at −4 and −2 positions (C-4G and G-2S) (*panel* B, JE-LSS-M), by shortening the n-region (*panel* C, JE-SS-ORI), or by a combination of both modifications (*panel* D, JE-SS-M).

TABLE 3. Neutralizing antibody (Nt) responses in mice immunized with different doses of the combined WN and JE virus DNA vaccines

	pCBWN + pCBJE1-14 [dose per plasmid (μg)]				pCB control
	100 + 100	40 + 40	20 + 20	10 + 10	(100 μg/ plasmid)
Percentage of mice with Nt:					
WN virus/JE virus	100/100	100/70	70/0	60/0	0/0
Range of $PRNT_{90}$ titer:					
WN virus	1:320–1:80	1:80–1:20	1:80–<1:10	1:20–<1:10	<1:10
JE virus	1:40–1:10	1:10–<1:10	<1:10	<1:10	<1:10

NOTE: Groups of 10, 3-week-old, female ICR outbred mice were i.m. injected with a single dose of combined plasmid DNAs as indicated. The serum specimens collected 12 weeks after immunization were assayed by the plaque-reduction neutralization test (PRNT). The end-point titers against JE and WN virus were calculated based on the 90% plaque reduction using JE virus (strain SA-14) and WN virus (strain NY-6480), respectively.

because a single i.m. injection of pCBWN DNA not only induced a protective immunity, but also prevented WN virus infection in mice and horses.

FUTURE DIRECTIONS

Efficient synthesis of the immune-dominated protective antigen(s) in its native conformation is indispensable for the development of an effective DNA vaccine. Thus, designing an optimal signal sequence at the N-terminus of the prM gene is the key element and deserves more attention to achieve this objective. Virus-encoded signal sequence is, by no means, the only optimal signal peptide available. Using the signal peptide encoded in the pCBJE1-14 plasmid as an example, the signal sequence probability can be improved by shortening the n-region, altering the c-region sequence, or a combination of both modifications (FIG. 2). We have used the shortened version of JE virus signal peptide for the expression of WN virus prM and E genes.[35] Dose titration studies by single i.m. inoculation indicated that the pCBWN was at least 2- to 4-fold more immunogenic than pCBJE1-14 in mice (G-J. Chang, unpublished observation). Increased immunogenicity of the pCBWN vaccine may correlate with the observation that secretion of WN virus proteins by pCBWN plasmid was more efficient than its JE virus counterpart.

Use of multivalent and/or combination vaccines designed to immunize against multiple flaviviruses is also a promising area of development. The first step to achieve this goal is the construction of monovalent vaccine components that include all important human pathogens, such as YF, four serotypes of DEN, JE, WN, SLE, and TBE (RSSE and CEE) viruses. Use of the combination vaccine to protect immunized animals from RSSE and CEE virus infections was first demonstrated by Schmaljohn and coworkers.[27,54] Preliminary data from our group also demonstrated that i.m. injection of the combined pCBJE1-14 and pCBWN DNA vaccines induced

JE virus– and WN virus–specific Nt antibodies in mice (TABLE 3; G-J. Chang, unpublished results). Each monovalent component, constructed using an expression vector that has identical transcriptional and translational regulators, needs to be tested in the same model system to ensure its vaccine potential. A combination vaccine cocktail can then be formulated specifically for a particular geographic region. The vaccine cocktail for tropical and subtropical Asia should include four serotypes of DEN, WN, and JE virus vaccines. Likewise, four serotypes of DEN, WN, and YF virus vaccines and four serotypes of DEN, Rocio, and YF virus vaccines are included in the cocktail for Africa and Latin America, respectively.

REFERENCES

1. KUNO, G., G.J. CHANG, K.R. TSUCHIYA, *et al.* 1998. Phylogeny of the genus *Flavivirus*. J. Virol. **72:** 73–83.
2. BRANDT, W.E. 1990. From the World Health Organization: development of dengue and Japanese encephalitis vaccines. J. Infect. Dis. **162:** 577–583.
3. CHAMBERS, T.J., T.F. TSAI, Y. PERVIKOV & T.P. MONATH. 1997. Vaccine development against dengue and Japanese encephalitis: report of a World Health Organization meeting. Vaccine **15:** 1494–1502.
4. GUIRAKHOO, F., R. WELTZIN, T.J. CHAMBERS, *et al.* 2000. Recombinant chimeric yellow fever–dengue type 2 virus is immunogenic and protective in nonhuman primates. J. Virol. **74:** 5477–5485.
5. MONATH, T.P., I. LEVENBOOK, K. SOIKE, *et al.* 2000. Chimeric yellow fever virus 17D–Japanese encephalitis virus vaccine: dose-response effectiveness and extended safety testing in rhesus monkeys. J. Virol. **74:** 1742–1751.
6. GUIRAKHOO, F., Z.X. ZHANG, T.J. CHAMBERS, *et al.* 1999. Immunogenicity, genetic stability, and protective efficacy of a recombinant, chimeric yellow fever–Japanese encephalitis virus (ChimeriVax-JE) as a live, attenuated vaccine candidate against Japanese encephalitis. Virology **257:** 363–372.
7. MONATH, T.P., K. SOIKE, I. LEVENBOOK, *et al.* 1999. Recombinant, chimaeric live, attenuated vaccine (ChimeriVax) incorporating the envelope genes of Japanese encephalitis (SA14-14-2) virus and the capsid and nonstructural genes of yellow fever (17D) virus is safe, immunogenic, and protective in non-human primates. Vaccine **17:** 1869–1882.
8. BUTRAPET, S., C.Y. HUANG, D.J. PIERRO, *et al.* 2000. Attenuation markers of a candidate dengue type 2 vaccine virus, strain 16681 (PDK-53), are defined by mutations in the 5′-noncoding region and nonstructural proteins 1 and 3. J. Virol. **74:** 3011–3019.
9. HUANG, C.Y., S. BUTRAPET, D.J. PIERRO, *et al.* 2000. Chimeric dengue type 2 (vaccine strain PDK-53)/dengue type 1 virus as a potential candidate dengue type 1 virus vaccine. J. Virol. **74:** 3020–3028.
10. BRAY, M. & C.J. LAI. 1991. Construction of intertypic chimeric dengue viruses by substitution of structural protein genes. Proc. Natl. Acad. Sci. USA **88:** 10342–10346.
11. LIU, H-M., D-P. ZHENG, L-B. ZHANG, *et al.* 2000. Molecular evolution of a type 1 wild-vaccine poliovirus recombinant during widespread circulation in China. J. Virol. **74:** 11153–11161.
12. GUILLOT, S., V. CARO, N. CUERVO, *et al.* 2000. Natural genetic exchanges between vaccine and wild poliovirus strains in humans. J. Virol. **74:** 8434–8443.
13. BERKHOUT, B., K. VERHOEF, J.L.B. VAN WAMEL, *et al.* 1999. Genetic instability of live, attenuated human immunodeficiency virus type 1a vaccine strains. J. Virol. **73:** 1138–1145.
14. WANG, S., X. LIU, K. FISHER, *et al.* 2000. Enhanced type I immune response to a hepatitis B DNA vaccine by formulation with calcium- or aluminum phosphate. Vaccine **18:** 1227–1235.
15. DONNELLY, J.J., J.B. ULMER, J.W. SHIVER, *et al.* 1997. DNA vaccines. Annu. Rev. Immunol. **15:** 617–648.

16. RICE, C.M., E.M. LENCHES, S.R. EDDY, *et al.* 1985. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. Science **229:** 726–733.
17. CHAMBERS, T.J., C.S. HAHN, R. GALLER, *et al.* 1990. Flavivirus genome organization, expression, and replication. Annu. Rev. Microbiol. **44:** 649–688.
18. STADLER, K., S.L. ALLISON, J. SCHALICH, *et al.* 1997. Proteolytic activation of tick-borne encephalitis virus by furin. J. Virol. **71:** 8475–8481.
19. KAUFMAN, B.M., P.L. SUMMERS, D.R. DUBOIS, *et al.* 1989. Monoclonal antibodies for dengue virus prM glycoprotein protect mice against lethal dengue infection. Am. J. Trop. Med. Hyg. **41:** 576–580.
20. BRANDRISS, M.W., J.J. SCHLESINGER, E.E. WALSH, *et al.* 1986. Lethal 17D yellow fever encephalitis in mice: I. Passive protection by monoclonal antibodies to the envelope proteins of 17D yellow fever and dengue 2 viruses. J. Gen. Virol. **67:** 229–234.
21. CECILIA, D., D.A. GADKARI, N. KEDARNATH, *et al.* 1988. Epitope mapping of Japanese encephalitis virus envelope protein using monoclonal antibodies against an Indian strain. J. Gen. Virol. **69:** 2741–2747.
22. ZHANG, M.J., M.J. WANG, S.Z. JIANG, *et al.* 1989. Passive protection of mice, goats, and monkeys against Japanese encephalitis with monoclonal antibodies. J. Med. Virol. **29:** 133–138.
23. KREIL, T.R., E. MAIER, S. FRAISS, *et al.* 1998. Neutralizing antibodies protect against lethal flavivirus challenge, but allow for the development of active humoral immunity to a nonstructural virus protein. J. Virol. **72:** 3076–3081.
24. MASON, P.W., S. PINCUS, M. FOURNIER, *et al.* 1991. Japanese encephalitis virus-vaccinia recombinants produce particulate forms of the structural membrane proteins and induce high levels of protection against lethal JEV infection. Virology **180:** 294–305.
25. KONISHI, E. & P.W. MASON. 1993. Proper maturation of the Japanese encephalitis virus envelope glycoprotein requires cosynthesis with the premembrane protein. J. Virol. **67:** 1672–1675.
26. PHILLPOTTS, R.J., K. VENUGOPAL & T. BROOKS. 1996. Immunisation with DNA polynucleotides protects mice against lethal challenge with St. Louis encephalitis virus. Arch. Virol. **141:** 743–749.
27. SCHMALJOHN, C., L. VANDERZANDEN, M. BRAY, *et al.* 1997. Naked DNA vaccines expressing the prM and E genes of Russian spring-summer encephalitis virus and Central European encephalitis virus protect mice from homologous and heterologous challenge. J. Virol. **71:** 9563–9569.
28. ABERLE, J.H., S.W. ABERLE, S.L. ALLISON, *et al.* 1999. A DNA immunization model study with constructs expressing the tick-borne encephalitis virus envelope protein E in different physical forms. J. Immunol. **163:** 6756–6761.
29. RAVIPRAKASH, K., T.J. KOCHEL, D. EWING, *et al.* 2000. Immunogenicity of dengue virus type 1 DNA vaccines expressing truncated and full length envelope protein. Vaccine **18:** 2426–2434.
30. KOCHEL, T., S.J. WU, K. RAVIPRAKASH, *et al.* 1997. Inoculation of plasmids expressing the dengue-2 envelope gene elicits neutralizing antibodies in mice. Vaccine **15:** 547–552.
31. LIN, Y.L., L.K. CHEN, C.L. LIAO, *et al.* 1998. DNA immunization with Japanese encephalitis virus nonstructural protein NS1 elicits protective immunity in mice. J. Virol. **72:** 191–200.
32. KONISHI, E., M. YAMAOKA, *et al.* 1998. Induction of protective immunity against Japanese encephalitis in mice by immunization with a plasmid encoding Japanese encephalitis virus premembrane and envelope genes. J. Virol. **72:** 4925–4930.
33. CHANG, G.J., A.R. HUNT & B. DAVIS. 2000. A single intramuscular injection of recombinant plasmid DNA induces protective immunity and prevents Japanese encephalitis in mice. J. Virol. **74:** 4244–4252.
34. COLOMBAGE, G., R. HALL, M. PAVY, *et al.* 1998. DNA-based and alphavirus-vectored immunisation with prM and E proteins elicits long-lived and protective immunity against the flavivirus, Murray Valley encephalitis virus. Virology **250:** 151–163.
35. DAVIS, B.S., G-J.J. CHANG, B. CROPP, *et al.* 2001. West Nile virus recombinant DNA vaccine protects mouse and horse from virus challenge and expresses *in vitro* a non-

infectious recombinant antigen that can be used in enzyme-linked immunosorbent assays. J. Virol. **75:** 4040–4047.
36. FLEETON, M.N., P. LILJESTROM, B.J. SHEAHAN, *et al.* 2000. Recombinant Semliki Forest virus particles expressing louping ill virus antigens induce a better protective response than plasmid-based DNA vaccines or an inactivated whole particle vaccine. J. Gen. Virol. **81:** 749–758.
37. SCHALICH, J., S.L. ALLISON, K. STIASNY, *et al.* 1996. Recombinant subviral particles from tick-borne encephalitis virus are fusogenic and provide a model system for studying flavivirus envelope glycoprotein functions. J. Virol. **70:** 4549–4557.
38. FYNAN, E.F., R.G. WEBSTER, D.H. FULLER, *et al.* 1993. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. Proc. Natl. Acad. Sci. U.S.A. **90:** 11478–11482.
39. SCHMALJOHN, C., D. CUSTER, L. VANDERZANDEN, *et al.* 1999. Evaluation of tick-borne encephalitis DNA vaccines in monkeys. Virology **263:** 166–174.
40. MIR, L.M., M.F. BUREAU, J. GEHL, *et al.* 1999. High-efficiency gene transfer into skeletal muscle mediated by electric pulses. Proc. Natl. Acad. Sci. USA **96:** 4262–4267.
41. PORTER, K.R., T.J. KOCHEL, S.J. WU, *et al.* 1998. Protective efficacy of a dengue 2 DNA vaccine in mice and the effect of CpG immuno-stimulatory motifs on antibody responses. Arch. Virol. **143:** 997–1003.
42. KONISHI, E., M. YAMAOKA, I. KURANE, *et al.* 2000. A DNA vaccine expressing dengue type 2 virus premembrane and envelope genes induces neutralizing antibody and memory B cells in mice. Vaccine **18:** 1133–1139.
43. KONISHI, E., M. YAMAOKA, I. KURANE, *et al.* 2000. Japanese encephalitis DNA vaccine candidates expressing premembrane and envelope genes induce virus-specific memory B cells and long-lasting antibodies in swine. Virology **268:** 49–55.
44. VOGEL, F. & N. SARVER. 1995. Nucleic acid vaccines. Clin. Microbiol. Rev. **8:** 406–410.
45. CAVENER, D.R. & S.C. RAY. 1991. Eukaryotic start and stop translation sites. Nucleic Acids Res. **19:** 3185–3192.
46. KOZAK, M. 1997. Recognition of AUG and alternative initiator codons is augmented by G in position +4, but is not generally affected by the nucleotides in positions +5 and +6. EMBO J. **16:** 2482–2492.
47. ALLISON, S.L., C.W. MANDL, C. KUNZ, *et al.* 1994. Expression of cloned envelope protein genes from the flavivirus tick-borne encephalitis virus in mammalian cells and random mutagenesis by PCR. Virus Genes **8:** 187–198.
48. AN, L.L., F. RODRIGUEZ, S. HARKINS, *et al.* 2000. Quantitative and qualitative analyses of the immune responses induced by a multivalent minigene DNA vaccine. Vaccine **18:** 2132–2141.
49. VON HEIJNE, G. 1985. Signal sequences: the limits of variation. J. Mol. Biol. **184:** 99–105.
50. LOBIGS, M. 1993. Flavivirus premembrane protein cleavage and spike heterodimer secretion require the function of the viral proteinase NS3. Proc. Natl. Acad. Sci. USA **90:** 6218–6222.
51. NIELSEN, H. & A. KROGH. 1998. Prediction of signal peptides and signal anchors by a hidden Markov model. Proc. Int. Conf. Intell. Syst. Mol. Biol. **6:** 122–130.
52. SAKAGUCHI, M., R. TOMIYOSHI, T. KUROIWA, *et al.* 1992. Functions of signal and signal-anchor sequences are determined by the balance between the hydrophobic segment and the N-terminal charge. Proc. Natl. Acad. Sci. USA **89:** 16–19.
53. HUNT, A.R., B.C. CROPP & G.-J.J. CHANG. 2001. A recombinant particulate antigen of Japanese encephalitis virus produced in stably-transformed cells is an effective non-infectious antigen and subunit immunogen. J. Virol. Methods **97:** 133–149.
54. SCHMALJOHN, C., D. CUSTER, L. VANDERZANDEN, *et al.* 1999. Evaluation of tick-borne encephalitis DNA vaccines in monkeys. Virology **263:** 166–174.